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CHRONIC PANCREATITIS, PAIN, AND ANXIETY IN AN ALCOHOL AND HIGH FAT MOUSE MODEL

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Dr. Karin Westlund High, Major Professor

Dr. John Watkins, Director of Graduate Studies

CHRONIC PANCREATITIS, PAIN, AND ANXIETY IN AN ALCOHOL AND HIGH FAT MOUSE MODEL

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Gerontology at the University of Kentucky

By

Tiffanie Clinkinbeard

Lexington, KY

Co-Directors: John F. Watkins, Professor of Gerontology and Karin Westlund High, Professor of Physiology

ABSTRACT OF DISSERTATION

CHRONIC PANCREATITIS, PAIN, AND ANXIETY IN AN ALCOHOL AND HIGH FAT MOUSE MODEL

Homeodynamic space (HDS) shrinks as vulnerability increases with aging and repeated damage to the cells. HDS is lost in alcoholic pancreatitis patients due to overconsumption of alcohol, smoking, and high fat diets. Etiologically relevant animal models for study of chronic pancreatitis (CP) are needed. In order to begin filling this gap a central purpose of this dissertation research was to examine relationships between the alcohol and high fat diet (AHF) and pancreatitis with attention to hypersensitivity and anxiety-like behaviors. The AHF diet induced pancreatitis described here etiologically mimics human risk factors of AHF consumption for advancement to alcoholic CP.

In this study one group of mice was fed long term with a diet of high fat and alcohol for comparison with a group fed normal chow. Mice consumed a liquid diet containing 6% alcohol and a high fat supplement ad libitum over a period of five months. Each group was evaluated for heat and mechanical hypersensitivity, and histology indicative of CP.

The association of pancreatitis pathology with anxiety has been understudied. Anxiety, like pain, is useful as a transient state but when anxiety is prolonged it is termed a disorder. Anxiety is often comorbid with pain and depression. Therefore, it is important to determine anxiety in mice with CP histology.

This model was characterized for the interaction of pancreatitis histology, as well as persisting pain-, anxiety-, and fear-like behaviors. The AHF diet mice developed hypersensitivity, demonstrated anxiety-like behaviors, and showed concurrent histology consistent with CP. Nontransgenic mouse models where pancreatitis is induced only by a combination of ad libitum liquid food with added alcohol and lard supplementation do not currently exist, nor has an in-depth study of anxiety-like behaviors been conducted in this mouse model. This dissertation research addresses this knowledge gap.

KEYWORDS: Pain, Pancreatitis Mouse Model, Anxiety, Alcohol, High Fat, Fear

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May 20, 2016

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CHRONIC PANCREATITIS, PAIN, AND ANXIETY IN AN ALCOHOL AND HIGH FAT MOUSE MODEL

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DEDICATION

To my family

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CHAPTER ONE: Introduction and Background

1 INTRODUCTION

Eight in 100,000 people in the United States are diagnosed annually with painfully debilitating chronic pancreatitis (CP) (Yadav and Lowenfels, 2013) and up to 50% of diagnosed individuals live up to 20 years with CP (Braganza et al., 2011). CP is described as an unresolvable inflammation of the pancreas accompanied by debilitating abdominal pain, nausea, vomiting, exocrine and endocrine insufficiency, as well as pathological changes impeding normal function that include fibrosis and ductal strictures. Alcoholic chronic pancreatitis is irreversible by medical means other than surgical removal of the pancreas, which may be effective. Suicide is more common in the pancreatitis patient population (4.9%) than in the general population (1.2%) (Nojgaard, 2010).

The National Commission on Digestive Diseases (NCDD) under the umbrella of the National Institute of Diabetes and Digestion and Kidney Diseases (NIDDK), value the production of better animal models to further the study of alcoholic pancreatitis (Witt et al., 2007). Pain-like behaviors in CP animal models are a crucial area to research and have also been called for (Reed, 2014) (Refer to Table 1 for definitions of pain terminology). Nontransgenic mouse models where pancreatitis is induced only by a combination of ad libitum liquid food with added alcohol and lard supplementation do not currently exist, nor has an in-depth study of anxiety-like behaviors been conducted in this mouse model. This dissertation research addresses this knowledge gap.

Current rodent models of pancreatitis induced by injected chemical irritants such as cerulein or dibutyltin dichloride (DBTC) have proven fruitful for studies of acute pancreatitis. Cerulein injections expose the pancreas to supraphysiologic cholecystokinin (CCK) levels,

which are not associated with the development of CP in humans, but do produce acute pancreatitis-like symptoms in mice (Reed, 2014). Cerulein treatment, a common and reproducible method for induction of acute pancreatitis (AP) in mice, causes recurrent damage to the pancreas as repeated injections are given, mimicking the cycle of damage to the pancreas by repeated human alcohol use (Reed, 2014). DBTC treatment is a less used method as it is not highly reproducible, causing acute disease development at a rate of about 33% of treated animals in the hands of some investigators (Reed, 2014). In addition, DBTC does not cause CP in humans (Reed, 2014). The cerulein and DBTC models, while useful for study of acute pancreatitis, do not provide the range of risk factors seen in clinic for CP.

Table 1. Definition of Pain Terms*

- Pain - an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.
- Acute Pain - pain due to illness or injury that resolves and heals before three months time.
- Chronic Pain – pain due to damage, injury, or unknown causes lasting at least 3 months. Chronic pain is ongoing or recurring and may never resolve.
- Allodynia - an abnormally increased painful response to an innocuous stimulus. An example is heightened pain in sunburned skin as clothes brush against it.
- Analgesia - absence of pain in response to stimulation which would normally be painful.
- Hyperalgesia – pain is exaggerated and prolonged in response to noxious stimuli. (Latremoliere & Woolf, 2009)

*Definitions from http://www.iasp-pain.org/AM/Template.cfm?Section=Pain_Definitions#Hyperalgesia accessed 12/9/13, or http://theacpa.org/Glossary accessed 1/8/16

Drinking alcohol can reduce anxiety, relieve stress, and facilitate relaxation in people (Gilman et al., 2008). These reasons and others associated with the effects of alcohol can lead to

the overuse of alcohol over time and subsequent pancreatic damage (See Table 2 for definition of anxiety terminology). Damage to the body from alcohol reduces physiological reserve and impacts the ability to maintain health. Physiological reserve is a measure of the ability of an organism to adapt physically to stressful stimuli using reserve capacity (Kooman et al., 2013). This alcohol-induced damage works in an additive manner to drive losses of physiological reserve and health beyond those experienced in normative aging.

Table 2. Definition of Anxiety Terms

- Anxiety – A psychological, physiological, and behavioral state induced in animals and humans by a threat to wellbeing or survival, either actual or potential. It is characterized by increased arousal, expectancy, autonomic and neuroendocrine activation, and specific behavior patterns (Steimer, 2002).
- Fear - a motivational state aroused by specific stimuli that give rise to defensive behavior or escape. (McFarland et. Al, 1987)
- Escape – characterized by "flight" in the "flight or fight" physiological reaction to a stressor perceived as dangerous.
- Contextual fear – fear present in a certain context due to previous negative conditioning experienced while within the specific context. (Campos et. Al, 2013)
- Stress Induced Anxiety (SIA) – an in-built mammalian pain suppression response that occurs during or following exposure to a stressful or fearful stimulus. (Butler & Finn, 2009)

Anxiety is a factor in increased life stress and has been shown to accelerate the progression and severity of diseases such as cardiovascular disease (CVD) (Krantz and McCeney, 2002), HIV (Leserman et al., 2002), and depression (Hammen, 2005). Chronic stress alone, while a risk factor in the development of acute pancreatitis (Binker & Binker, 2014), has not been shown to cause pancreatitis. The reduction of anxiety is one possible reason for the consumption of more than four alcoholic drinks a day which increases the likelihood of developing CP (Kushner et al., 2005, Gilman et al., 2008, Irving et al., 2009). Production of a

mouse model that more fully follows etiological risk factors seen in human alcoholic CP provides a significant research tool for this disease, giving an etiologically more realistic view of the irreversible damage done to the pancreas of the CP patient by continuing alcohol overconsumption in the presence of a high fat diet.

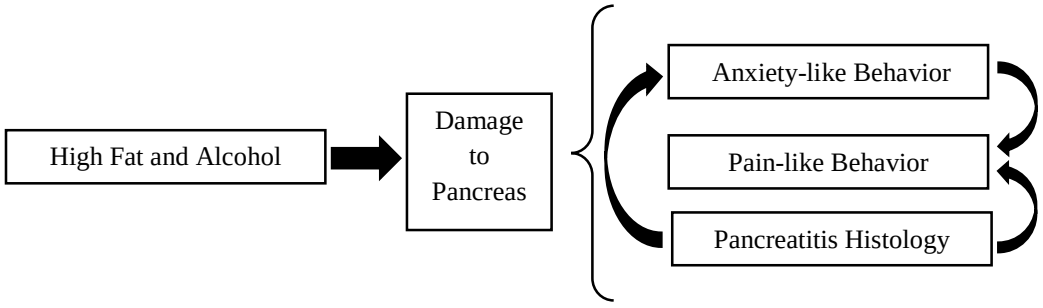

Figure 1. A diet high in fat and alcohol damages the pancreas. The interaction of pain-like behavior, pancreatitis histology, and anxiety-like behavior.

Etiologically relevant animal models for study of CP are needed. In order to begin filling this gap a central purpose of this dissertation research was to examine relationships between the AHF diet and pancreatitis with attention to hypersensitivity and anxiety-like behaviors. The alcohol and high fat diet induced pancreatitis described here more fully recapitulates human etiological risk factors of alcohol and high fat consumption for advancement to pancreatitis. This model was characterized for the interaction of the chronic pathological state, persisting pain-like behaviors, and anxiety-like behaviors. The research shows that feeding an alcohol and high fat diet leads to the development of CP, hypersensitivity, and model anxiety behaviors characterized using non-reflexive and reflexive testing. Pancreatitis histology, as well as quantitative and qualitative observation of anxiety behaviors, and increased pain-like behaviors indicated loss of physiological reserve in the AHF mice. Pain-like and anxiety-like behaviors in the mouse model mimic behavior seen in humans with CP.

2 PURPOSE

Current models of chemical induction of pancreatitis are well suited for the study of acute pancreatitis, however, alcoholic CP models have been called for by those prominent in the field. Mouse models showing induction of alcoholic CP in nontransgenic mice using only an ad libitum alcohol enriched liquid diet, with supplemental fat are not currently available. The purpose of this dissertation research was to develop an alcohol and high fat (AHF) diet induced mouse model of CP, while evaluating sensory hypersensitivity, anxiety- and fear-like behaviors to provide a reliable and robust mouse model for the study of human alcoholic chronic pancreatitis. It should be noted that scientists use animals such as mice to mimic human diseases for the purposes of furthering understanding of human disease through animal research. In this study a mouse model was developed that shows CP histology, heat and mechanical hypersensitivity, and anxiety-like behavior (Figure 1). This mouse model mimics the possible physical consequences of human overconsumption of alcohol and high calorie foods.

3 SPECIFIC AIMS

3.1 AHF Induced Alcoholic Chronic Pancreatitis Mouse Model

Specific Aim 1: To characterize pain-like behaviors and pancreatic histology in mice on an alcohol and high fat diet versus controls. Hypothesis: Mice on the AHF diet will display hyperalgesia and pancreatitis histology. Behaviors and histology were expected to conform to that seen in rats that developed CP on the AHF diet. AHF-fed rats develop hyperalgesia, increased pancreatic fibrosis, and damage to pancreatic cells and structures defined by further histological scoring. Pancreatic histology was assessed using Fast Green with Sirius Red counterstain of collagen fibrosis; other cellular morphology was also assessed. Testing included mechanical and thermal behavioral assays included the von Frey, hotplate, modified hotplate, and the smooth or rough textured mechanical plate.

3.2 Anxiety- and Fear-like Behaviors in the AHF Mouse Model

<u>Specific Aim 2</u>: To characterize anxiety- and fear-like behaviors in mice using non-reflexive testing. Hypothesis: Mice on the AHF diet will display higher anxiety- and fear-like behaviors than control mice in non-reflexive testing modalities. While rats respond in a quantifiable manner to tests such as the hotplate, mice are historically less reliable than rats using reflexive testing. For example, in the 50°C hotplate test mice did not show the typical threshold markers seen in rats such as licking the hind paw before the cut-off time. While these testing methods may appear to measure only sensitivity, sensitivity and anxiety-like/fear-like behaviors are interrelated. In this case reflexive testing, in addition to being unreliable with the research mice, also would not have allowed the depth of work with anxiety-like and fear-like behaviors. Mouse testing methods returning reliable results were developed using non-reflexive methods to optimize behavioral testing in this CP mouse model. The following assays were used to measure mouse fear-like and anxiety-like behaviors: plus maze, open field, place preference and escape, counts of grooming, counts of rearing, and number of uncued freeze events. Non-reflexive testing allowed for a more holistic view of the mouse study treatment groups, highlighting anxiety-like and fear-like behaviors present in the animal.

4 BACKGROUND

4.1 Pain Sensing

4.1.1 Cutaneous Pain Sensing

The ability to sense nociceptive stimuli is necessary in order for the organism to remove itself from the source of nociceptive stimuli and keep itself from future injury. In this way pain behaviors assist an animal in survival. Cutaneous nociception occurs through specialized nociceptors that are activated by noxious thermal, mechanical, or chemical stimuli (Woolf, 2004). Activation of nociceptive receptors or ion channels by noxious stimuli generates an

electrical signal. Nociception is composed of transduction, conduction, transmission, and perception. The receptor or ion channel transduces the stimuli into electrical current which becomes an action potential. Afferent nerves conduct the action potential to the spinal cord from the periphery. The action potential causes neurotransmitters to be transmitted from the central terminal across the synapse to the dorsal horn neurons (Scholz and Woolf, 2002). The nociceptive signal then travels to the brain through ascending pathways such as the spinothalamic tract. Perception occurs when the human brain receives the signal and perceives pain. An example of cutaneous nociceptive signaling occurs when a person gets a cut on their foot and inspects it for damage.

4.1.2 Fiber Type and Sensing

Three different fiber types, the Aβ, Aδ, and C fibers carry a wealth of information to the brain concerning the environment. Aβ nerve fibers have the largest diameter of the three, are well myelinated, and are the fastest to conduct signals. The Aβ fibers carry touch and pressure, while Aδ and C fibers carry nociceptive signals. Aδ nerve fibers have a smaller diameter than the Aβ, are lightly myelinated, and carry fast pain (described as sharp, lancing, prickly). C fibers have the smallest diameter of the three afferent nerve fiber types, with no myelination. C fibers carry slow pain (described as dull, burning, poorly localized).

4.1.3 Acute Pain

Acute pain results from damage to tissue. Injuries resulting in acute pain are often easily localized. A newly received cut on the foot is an injury that would cause acute pain. Acute pain resolves before three months' time as the damaged tissue heals (IASP, 2004, ACPA, 2016). Acute pain serves the evolutionally important function of warning of dangerous environments

and enabling protection against further damage (Scholz and Woolf, 2002). Unrelieved acute pain can develop into chronic pain.

4.1.4 Peripheral Sensitization

Acute pain can become chronic pain through the process of sensitization. When there is acute tissue damage the environment around the nociceptors changes. Inflammation occurs and chemical factors in the 'inflammatory soup' like chemokines and cytokines are released (Scholz and Woolf, 2002, Woolf, 2004). Some of the factors incite pain while others make nociceptors within their reach hypersensitive to stimuli.

Modulation of receptors or ion channels due to posttranslational changes causes sensitization. Chemical factors bind receptors on nociceptors located in the periphery (Scholz and Woolf, 2002, Woolf, 2004). Intracellular kinases bind to and activate nociceptive receptors and ion channels. Pain hypersensitivity becomes apparent as the threshold of activation due to painful stimuli decreases (Woolf, 2004).

4.1.5 Central Sensitization

The development of central sensitization is similar to that in peripheral sensitization and occurs in the dorsal horn. Posttranslational changes occur in secondary sensor neurons (Scholz and Woolf, 2002, Woolf, 2004). In the dorsal horn, neurotransmitter activity by the central terminals increases. Synaptic receptors like NMDA are phosphorylated. Hyperalgesia develops as synaptic receptors show increased responsiveness to sensory input (Woolf, 2004).

4.2 Chronic Pain and Health

4.2.1 Chronic Pain

Pain that was termed "acute" becomes chronic pain, by definition, after three months (IASP, 2004). Chronic pain generally arises from an injury or damage to the body but may have

no discernible physical cause. This pain may be present even in the absence of observed damage to tissue, for example in people with amputated limbs. Chronic pain can be continuous and unrelenting, or it may be intermittent. This type of long-lasting pain may never resolve. People in chronic pain experience physical, social, and mental decreases in function (Figure 2) (Fine, 2011). Pain in this sense encompasses physical pain such as that of CP, as well as mental pain manifestations such as anxiety. Chronic uncontrolled pain is exhausting and has great potential to decrease quality of life over the long term. Chronic pain can become debilitating (Fine, 2011, Mullady et al., 2011, Amann et al., 2013).

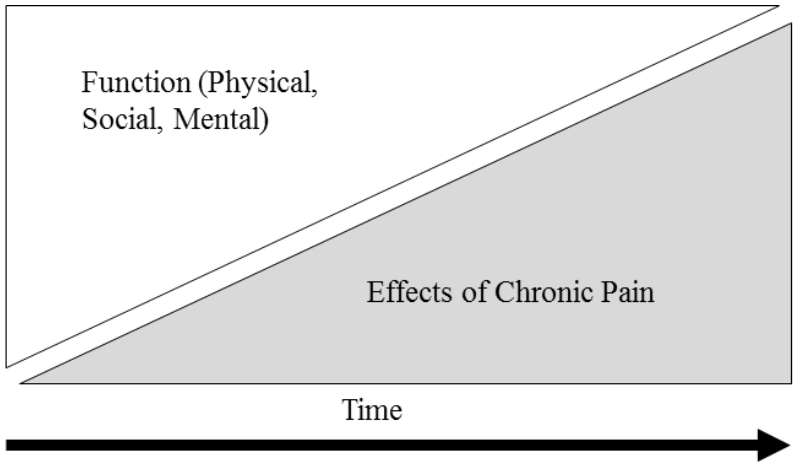

Figure 2. Physical function. As the effects of pain increase over time, physical function tends to decrease for the individual.

In humans physical disease progression and perceived physical pain can both cause and be caused by anxiety (Gibson and Helme, 2000, Chiu et al., 2005, Roy-Byrne et al., 2008, Lieb and Forsmark, 2009, Harvard, 2010). Perception of pain through a filter of chronic anxiety (Harvard, 2010) may increase the overconsumption of alcohol and high fat foods. Long-term consumption of high calorie foods and overconsumption of alcohol decreases physiological reserve.

The DNA between humans and mammalian species such as mice is well conserved, as are anatomy, and physiology (Dolensek et al., 2015, Nguyen et al., 2015). Nociceptive pathways

are similar in mice and in humans, with some minor differences. Characterizing as many factors as possible, such as anxiety- and fear-like behaviors, is a common practice in the development of a new animal model.

4.2.2 Homeodynamics & Homeostasis

Homeodynamics and homeostasis work together in determining the response of the body to stimuli. The term homeostasis describes an attempt by the body to continually return to one set point, that remains the same, such as temperature, while homeodynamics covers the changes in this set-point as the body attempts to adjust to changes affecting its ability to adapt, its homeodynamic space (Rattan, 2006). Homeostasis is more like a snapshot in time while homeodynamics is more like a longitudinal study as homeodynamics focuses on the entire lifespan. Homeodynamic space, a measure of fitness, shrinks during the aging process as cells and other biological systems become less adaptable (Rattan, 2008). Successful adaptation leads to diminishing of harmful effects on the body and a maintenance the homeodynamic space available to the individual. Unsuccessful adaptation leads to shrinking homeodynamic space and the inability to counter harmful stimuli as before (Rattan, 2006) (see Table 3). Shrinking homeodynamic space has its termination when the organism encounters harmful stimuli it can no longer defend against.

Table 3: Physical and Mental Pain

Physical Pain	Mental Pain
↑ physical pain	↑ mental pain (stress, anxiety, fear)
↓ ability to maintain health	↓ ability to maintain health
↓ homeodynamic space	↓ homeodynamic space

Physiological reserve (PR) is a measure of the ability of an organism to adapt to stressful stimuli using reserve capacity (Kooman et al., 2013) and can be used to understand homeodynamics (Rattan, 2013, 2014). Homeodynamic space is concerned with damage control, stress response, and adaptation (Rattan, 2013, 2014). These areas of homeodynamic space are impacted by the reserve capacity available for use. Body organs in general can lose about 70% of their physiological reserve before adequate function is impaired (Bortz, 2002). Impaired function of one or more body organs can decrease homeodynamic space. PR decreases over time in normative aging (Bortz, 2005). Continued stressful stimuli, in concert with normative aging likely decrease PR in an additive fashion over time (Bortz, 2005).

4.2.3 Homeodynamic Space and Pancreatitis

The adaptability of an organism's homeostatic set points to adjust over time with fluctuations in the environment is homeodynamics (Rattan, 2008). Shrinking homeodynamic space is apparent in the loss of adaptability of the pancreas to repeated insults by overconsumption of alcohol and high calorie foods over time. Lost PR in the pancreas in the presence of the AHF diet sets the stage for the development of pancreatitis. Alcohol and a high fat diet provide a negative stressful stimuli to the cells of the pancreas, which must respond to the stimuli and control damage resulting from it. Cells maintaining the function of the pancreas must respond to repeated damaging AHF. The continual presence of AHF toxins is likely to continually interrupt cellular pancreatic maintenance, leading to pancreatic damage through dysregulation of typical pancreatic repair mechanisms. Unhealthy pancreatic parenchyma experience a shrinking homeodynamic space, a decreasing ability to adapt to change (Rattan, 2006). A decrease in homeodynamic space leaves the organism open to greater detrimental

effects from stresses than previously experienced as adaptation to the stimuli is impaired. Alcoholic chronic pancreatitis could be the consequence of an inability to adapt to stimuli.

4.2.4 Human Adaption to the Loss of Homeodynamic Space

There are adaptations to the loss of homeodynamic space that humans can make that would be impossible for laboratory mice. Changes in homeodynamic space are diverse in origin and nature. For example, in some individuals with unusual genetics, their health will be much greater than the norm (Rajpathak et al., 2011). Smoking and even heavy drinking may not decrease their physiological reserve (Milman and Barzilai, 2015). Regardless of genetics some people may profit from a family or culture that is familiar with the needs of those in old age and can extend the lived years using family or cultural methods (checking in on someone, bringing meals, having the older person/couple move in with children, other techniques such as providing excellent paid healthcare to an older person in a nursing home).

There are indications that longevity is genetic and can be inherited (Milman and Barzilai, 2015). Part of the individual ability of someone to reach much older age may have to do with the understanding of the possibility of reaching older age, the changes that come with aging (so less ageism within the “tribe”, more understanding), and the needs of the older person for assistance and caregiving with each stage of older life, in both the individual (as they saw it growing up), and in the family. The average younger individual from a family where grandparents passed away early and there were few if any much older people around is less likely to understand older age and the changes that come with it than someone surrounded by long-lived family members throughout their life. The younger individual from a landscape barren of older people may have less ability and understanding of how to help an older person to maintain their homeodynamic space or even that they can do such a thing. Note that higher socioeconomic groups tend to live

longer (Crimmins, 2011), possibly due to the use of resources commonly available to higher socioeconomic groups such as time, financial resources, or social connections to allow better care of the older adult. Inequality in education, occupation, and health care accessibility are just a few possible causes for decreased life span and health in lower socioeconomic groups (Marmot, 2015).

An in depth review of the heterogeneity of aging, the attainment of longevity, and the many possible human factors leading to the maintenance of human homeodynamic space is beyond the scope of this dissertation. A detailed discussion of possible human social interventions would also be out of place in this description of research leading to the successful development and characterization of a mouse model of chronic pancreatitis.

4.3 Social Aspects of Alcoholic Chronic Pancreatitis

A number of factors can combine to contribute to loss of physiological reserve within the human body as well as within the pancreas itself. Often two or more of these endogenous or exogenous factors interact to provide “two hits” that allow pancreatitis to develop: inherited disease mutations, a diet high in fat, the overconsumption of alcohol, smoking, and chronic stress/anxiety.

One way to help add more years to life and more life to years is to better understand the complexity of how disease affects people, and study how to cure or at least temper the symptoms of painful diseases such as CP. There are indicators (Takano et al., 1992) that stress and anxiety can speed progression or severity of CP. There are also indications that counseling and other interventions that pay attention to the whole person, especially their mental state, can help decrease healthcare utilization (Madan et al., 2013).

In 2004, pancreatitis cost the United States $373.3 million in direct and indirect costs (Everhart and Ruhl, 2009). There are three main types of direct medical cost: doctor visits, hospitalization, and diagnostic tests (Sherman et al., 2001). Indirect costs include out-of-pocket expenses related to patient care, such as loss of wages due to illness or sacrifice of caregiver time; payments for prescription drugs; and copayments for healthcare (Houts et al., 1984, Sherman et al., 2001).

There are no causal cures for CP; behavioral modification can help, especially in acute pancreatitis. Madan (2013) shows that paying attention to the mental state of the pancreatitis patient is critical to controlling the disease. A possible method to control the progression of human AP and possibly CP could be to control the anxiety that Takano et al. (1992) has shown to speed the progression of CP in rats. A broader social change with cessation of blaming the sick, and with the provision of mental health services for those in need, with reversal of the stigma that can be attached to consulting a mental health professional could provide what is needed to help those at risk for developing, as well as those already suffering with pancreatitis.

4.4 Chronic Pancreatitis

CP is described as an unresolvable inflammation of the pancreas accompanied by debilitating abdominal pain, nausea, vomiting, exocrine and endocrine insufficiency, as well as pathological changes impeding normal function that include fibrosis and ductal strictures.

4.4.1 Classification of Pancreatitis by Etiology

Pancreatitis is classified according to the TIGAR-O system: toxic, idiopathic, genetic, autoimmune, recurrent acute, and obstructive (Kloppel, 2007, Nitsche et al., 2011). The dissertation research is specifically focused on the development of alcoholic chronic pancreatitis which falls within the toxic, more specifically a chronic toxic etiology.

Toxic sources for the development of pancreatitis include alcohol, exposure to cigarette smoke, hypercalcemia (hyperparathyroidism), hyperlipidemia (rare and controversial), chronic renal failure, medication such as phenacetin, and toxins such as organotin compounds (such as DBTC) (Etemad and Whitcomb, 2001).

An idiopathic disease has no known cause and this is true of idiopathic pancreatitis. The classification of pancreatitis as idiopathic is decreasing as an increasing number of genetic causes of pancreatitis are determined (Witt, 2007). Idiopathic pancreatitis is classified as Early Onset, Late Onset, Tropical, or Other (Etemad and Whitcomb, 2001). Early Onset idiopathic pancreatitis is diagnosed around 23 years, while Late Onset occurs around 62 years of age (Mullhaupt et al., 2005). Tropical pancreatitis is typically diagnosed around 22 years, progresses quickly, and shows extensive pancreatic damage (Balakrishnan et al., 2006).

A number of genetic causes of pancreatitis have been identified with more being discovered regularly. Early known genetic mutations predisposing people to pancreatitis were limited for a time to the cystic fibrosis transmembrane conductance regulator (CFTR), serine protease 1 (PRSS1), and the serine protease inhibitor, Kazal type 1 (SPINK1) mutations (Witt et al., 2007). Genetic pancreatitis symptoms can start as early as age 10 (Mullhaupt et al., 2005). They can be autosomal dominant or autosomal recessive (Etemad and Whitcomb, 2001). CFTR regulates bicarbonate secretion by the ducts and may be responsible for defective transport of inactive enzymes (Witt et al., 2006, Witt et al., 2007). There are a variety of PRSS1 mutations conferring gain of function, these can inhibit autolysis or increase auto-activation of trypsinogen (Teich et al., 2006, Witt et al., 2006). The SPINK1 mutation causes a loss in function in the inhibition of trypsinogen activation within the pancreas (Witt et al., 2007). CLDN2, as well as the following are more recent additions to the list of genetic mutations: CASR, CTRC, CTSB,

CTGI. Many genetic mutations have to do with changes in the inhibition and inactivation of the trypsin enzyme.

More heavy drinking men than women are afflicted with alcoholic CP. This is likely due in part to the influence of recently described X-linked polymorphism rs12688220, located near the human claudin-2 gene (Whitcomb et al., 2012). The rs12688220 single nucleotide polymorphism (SNP) is found in 50% of males with CP, but only 26% of males in the general population (MUSC Health, 2013). The increased percentage of CP in males with the rs12688220 SNP indicates that this SNP is a risk factor for CP.

Autoimmune pancreatitis (AIP) occurs when the body's immune system attacks its own pancreas. AIP responds to steroids (Kamisawa et al., 2008). The average age of diagnosis for AIP is 59 years old (Okazaki, 2001).

Recurrent Acute Pancreatitis (RAP) refers to pancreatitis that resolves then recurs. RAP causes reversible damage to the pancreas over a period of time. Doctors recommend abstinence from alcohol and quitting smoking to decrease the chances of recurrence. Users of Type 2 diabetes medications, sitagliptin and exenatide increase the risk of developing RAP by 6 times (Yadav and Lowenfels, 2013). Gallstones, for which age and female gender are a risk factor, are a common cause of acute pancreatitis (Yadav and Lowenfels, 2013).

Obstructive pancreatitis can occur for a number of reasons, one of which is tumorous obstruction of ducts (Kloppel, 2007).

4.4.2 Physiology of Chronic Pancreatitis

The pancreas is composed of both exocrine and endocrine cells. Pancreatic exocrine cells excrete digestive enzymes, while endocrine cells (known as Islets of Langerhans) serve homeostasis as they release glucagon and insulin hormones as needed into the bloodstream to

control blood sugar levels. The exocrine cells are known as acinar cells and are arranged around ducts through which unactivated digestive juices flow to the duodenum, become activated (trypsinogen becomes trypsin), and assist in the digestion of food. CP is characterized by unresolvable and painful inflammation of the pancreas as pancreatic enzymes such as trypsinogen, activate while still within the pancreas, leading to autodigestion of the pancreas (Hirota et al., 2006). The flow of unactivated digestive enzymes to the duodenum significantly decreases in pancreatitis as blocked ducts become unable to conduct enzymes out of the pancreas (Hirota et al., 2006). Autodigestion of the pancreas by prematurely activated trypsin causes collagen scar tissue to form; i.e. increase in pancreatic stellate cell (PSC) proliferation and fibrosis. This further decreases the function of the structures and cells within the pancreas. Histologically, an increase in fibrosis and activated PSC's indicates the chronic state of pancreatitis (Ammann and Mullhaupt, 2007, Erkan et al., 2009, Zhang et al., 2013, McIlwrath and Westlund, 2015, Zhang et al., 2015). Pancreatic insufficiency begins to affect both the exocrine and endocrine functions as CP progresses (Forsmark, 2013). The above is a general non-species specific recounting of the events that occur during the onset of chronic pancreatitis.

It is necessary to contrast both mouse and human pancreatic anatomy, and exocrine function. The endocrine function of the pancreas has not been shown to cause the development of alcoholic CP and will not be covered. The human pancreas is composed of a head, a body, and a tail. While it has distinct lobes, it is a solid and well-defined organ (Dolensek et al., 2015). The acinar cell is part of an acinus, which is responsible for moving secretions of the acinar cells into larger ducts. Pancreatic ducts drain from the acinus into larger ducts then finally into the one main pancreatic duct, which runs the length of the organ in humans (Dolensek et al., 2015). The

bile duct and the main pancreatic duct empty into the duodenum at the ampulla of Vater (Dolensek et al., 2015). The main duct in humans is adjacent to the bile duct.

The mouse pancreas is composed of three lobes: the splenic lobe (SL) (Watanabe et al., 1995), the duodenal lobe (DL), and the gastric lobe (GL) (Liu et al., 2010). The pancreas of the mouse is a solid as opposed to a hollow organ but is diffusely distributed in the mesentery of the small intestine (Bunnag et al., 1963). Each lobe of the three-lobed mouse pancreas has a main duct (splenic, duodenal, and gastric). The gastric duct drains into the splenic duct, which meets the bile duct, then empties into the ampulla of Vater. The duodenal duct separately meets the bile duct which empties at the duodenum into the ampulla of Vater (Lacy and Kostianovsky, 1967). The main ducts in the mouse pancreas join the bile duct before reaching the duodenum.

A comparison of human and mouse pancreatic anatomy, exocrine function, and tissue level similarities is necessary. The SL is like the human pancreatic body and tail (Watanabe et al., 1995), the DL is like the head, and the GL is like the pyramidal process in the pancreas of humans (Dolensek et al., 2015). The mouse pancreas has three “main” ducts, likely an adaptation to its more diffuse nature. The presence of more ducts to drain the mouse pancreas would be analogous to the larger ducts leading to the main duct in humans. Acinus are present in both human and mouse pancreas and serve the same general purpose, to provide enzymes to digest chyme. That human and mouse ducts are differently married to their respective pancreatic areas is likely not a critical factor in the development of CP because the arrangement of ducts in the mouse pancreas would be analogous to that in the human pancreas and would be expected to affect the end use of the pancreatic secretions in the same general way in both species. The development of CP begins when harmful stimuli affect the acinar cell. Dolensek (2015) in a

paper describing the similarities and differences inherent in mouse and human pancreas, mentions little difference between the individual acinar cells and the acinus.

In summary, while there are differences between the mouse and human pancreas, except for the few key differences notes below, they would be expected to have little impact on the development of CP and the use of this AHF diet to induce CP. The main difference between the AHF diet in humans versus mice follows: the distinctiveness and small surface area to volume ratio of the human pancreas versus the diffuse nature and large mass to volume ratio of mouse pancreas to mouse body could lead to faster alcohol exposure in the pancreas in mice relative to humans. Neuropathic changes such as the sprouting of nerves with continued damage to the pancreas would be expected to increase pancreatic nociceptive capability at a faster rate in the mouse. The differences mentioned in this paragraph are more likely to affect speed of progression of CP than the development of CP.

The factors below combine to make a compelling use for mouse models that mimic human diseases, but do not fully recapitulate them (Malina, 2010, Dolensek et al., 2015, Nguyen et al., 2015). Mouse models allow for research that would be unethical in humans (Nguyen et al., 2015). Comprehensive understanding and tools for modification of the mouse genetic code make the mouse useful as a genetic tool (Nguyen et al., 2015). Mice conveniently require relatively low maintenance, reproduce at a high rate, and have a short life-cycle (Malina, 2010, Nguyen et al., 2015). Mice are omnivorous and can consume diets as similar in composition to human diets as possible (Nguyen et al., 2015). Herbivorous animals are less likely to consume animal fat than are mice, making them less useful as AHF research model animals. Inbred mouse models provide a homogenous genetic background (Nguyen et al., 2015). While mouse/human genetic conservation is generally high (Malina, 2010, Nguyen et al., 2015), there are genetic differences

between the species, they are genetically similar but not exactly similar. In a similar manner, mouse anatomy and physiology is similar to humans but not exact (Nguyen et al., 2015). Laboratory conditions allow control of many factors in the mouse (Nguyen et al., 2015). Humans cannot be subjected to the same kind of control over diet and environment as mice can and this introduces additional variables into human experiments due to human heterogeneity.

4.4.3 Epidemiology of Chronic Pancreatitis

Alcoholic chronic pancreatitis is described as an unresolvable inflammation of the pancreas accompanied by debilitating abdominal pain, nausea, vomiting, exocrine and endocrine insufficiency, as well as pathological changes impeding normal function that include fibrosis and ductal strictures.

Alcohol overconsumption increases the risk of developing alcoholic CP, as does smoking. Alcohol has a 40% attributable risk in the development of CP (Frulloni et al., 2009), with 70% of those with CP showing alcoholic etiology (Martinez et al., 2004, Siech et al., 2009). Smokers have two to three times as much chance of developing CP as do nonsmokers (Yadav and Lowenfels, 2013).

The following suggests that a high fat diet increases the amount of fibrosis present in the pancreas. A high fat diet has been shown in rats to activate pancreatic stellate cells (PSC) and cause an increase in alpha smooth muscle actin (α-SMA, a marker for fibrosis) in the pancreas (Zhang et al., 2008, Zhang et al., 2015). Collagen is closely associated with α-SMA in CP (Haber et al., 1999). Fibrosis is composed of collagenous material like collagen 1.

Humans who eat a high fat diet are more likely to develop CP. Indeed, the contributions of a high fat diet in humans are expected to provide one of the two “hits” needed to develop alcoholic CP. Note that the obese have twice the risk of developing CP (Martinez et al., 2004).

Kentucky is ninth in the nation for obesity, with an obesity rate of 36% in 45-64 year olds in Kentucky (RWJF, 2013). Kentucky has a high percentage of obese people who could be at risk of developing chronic pancreatitis.

Scientists can use a variety of models, including models that use from biomolecules up to the whole animal to tease out the mechanisms, signaling pathways, and other characteristics of a disease such as chronic pancreatitis. These animal models are used to mimic human disease. As a common practice, scientists compare data collected from different mammalian models as well as humans in the same manuscript. Because rat, mouse, and human genomes are genetically conserved, mentioning how CP has been shown to work in rats, while adding how CP has been shown to or is expected to work in humans, gives justification to the production of a mouse model of CP using the AHF diet.

4.4.4 Risk Factors

The development of CP in humans has multiple possible contributing risk factors. Though many non-physical factors could contribute to the development of CP the risk factors of alcohol and obesity are the focus of this research. One risk factor is the sensitizing effect of alcohol on the pancreatic cells (Forsmark, 2013). Alcohol combines with other factors such as a high fat diet to cause pancreatic damage. People with a history of drinking heavily have four times the risk of developing CP than those that do not (Yadav et al., 2007b). Note that as a person ages their tolerance for alcohol declines so that the same amount of alcohol they used to drink becomes an unhealthy amount over time (NIA, 2011). High alcohol intake has also been associated with hypertriglyceridemia, another risk factor for pancreatitis (Klop et al., 2013).

More heavy drinking men than women develop alcoholic CP (Lankisch et al., 2002, Yadav et al., 2011a). This is likely due to the influence of recently described X-linked

polymorphism rs12688220, located near the claudin-2 gene (Whitcomb et al., 2012). The rs12688220 single nucleotide polymorphism (SNP) is found in 50% of males with CP, but only 26% of males in the general population (MUSC Health, 2013). The increased percentage of CP in males with the rs12688220 SNP indicates that this SNP is a risk factor for CP. Other mutations result in CP, these are: CFTR, SPINK1, and PRSS1 (Witt et al., 2007) as well as CTRC, and GGT1.

In 2004, pancreatitis cost the United States $373.3 million in direct and indirect costs (Everhart and Ruhl, 2009). There are three main types of direct medical cost: doctor visits, hospitalization, and diagnostic tests (Sherman et al., 2001). Indirect costs include out-of-pocket expenses related to patient care, such as loss of wages due to illness or sacrifice of caregiver time; payments for prescription drugs; and copayments for healthcare (Houts et al., 1984, Sherman et al., 2001).

4.4.5 The Disposable Soma Theory

The theory of disposable soma, proposed by Medawar (1952), is relevant to the common diagnosis period of CP at 40 to 50 years of age (Whitcomb et al., 2008). The theory of disposable soma suggests that natural selection is focused on maintaining health through the reproductive years in order to maximize the number of offspring. Once the bulk of reproduction is complete the body, the soma, is disposable, and natural genetic selection for fitness is neutral to the gradual loss of physiological reserve over time in the post reproductive organism (Kirkwood and Holliday, 1979, Charlesworth, 2000, Kirkwood, 2005). Natural selection includes genetic selection for high health during reproductive years. Human reproduction was historically complete or nearly complete by the mid 40’s during which alcoholic chronic pancreatitis is diagnosed. There was no reason to select for those without genetics that predisposed to CP

because the development of CP did not interfere with reproductive fitness. Note that across history survival to the mid 40's was not assured.

According to the disposable soma theory once reproduction is completed body systems and individual organs such as the pancreas begin to lose PR. With age the human pancreas shows fatty infiltration, some fibrosis, and a widening of the main pancreatic duct (Glaser and Stienecker, 2000, Wei, 2000). While the aging pancreas shows a gradual loss of physiological reserve over time, a pancreas with increased continuous damage due to etiologically determined factors in the development of CP such as alcohol would be expected to display much higher parenchymal fibrosis, along with other indicators of damage such as calcifications (Etemad and Whitcomb, 2001).

4.4.6 The Theory of Waste Accumulation

The theory of waste accumulation (Kirkwood, 1997) is apparent in the discussion of chronic pancreatitis. In normal function the quiescent pancreatic stellate cells (PSC), vitamin-A containing star-shaped support cells, lay down extracellular matrix (ECM) which is later removed. This process heals damage to the pancreas.

Damage to the pancreas activates the normally quiescent PSC to repair damage (Apte et al., 2012, Means, 2013). The Vitamin-A droplet of quiescent PSC's is lost with activation as the PSC take on a myofibroblast-like appearance (Bachem et al., 1998, Apte et al., 2012). In CP the activated PSC synthesize and deposit excessive ECM to resolve damage however, surplus ECM is not degraded (Apte et al., 2012). Dysregulation of ECM removal by the PSC leads to the accumulation of ECM. ECM presents as fibrosis and interferes with pancreatic function. The buildup of ECM decreases the physiological reserve of the pancreas, lowering its functional ability. Over time, this decreases homeodynamic space.

4.4.7 Development of Pain in Alcoholic Chronic Pancreatitis

The pain report in pancreatitis patients increases over time as the disease progresses from acute pancreatitis to CP. Acute pancreatitis pain tends to subside for a period of time as acute pancreatitis is indicative of reversible damage to the pancreas. AP risk of progression to alcoholic chronic pancreatitis depends on changes in drinking habits following the initial attack (Takeyama 2009). Takeyama et al. (2009) found that abstainers progressed to alcoholic CP in 14% of cases while those who continued habits of heavy drinking after AP diagnoses went on to develop alcoholic CP in 41% of cases. The pattern of developing AP and progressing to CP is important in pain development in CP because pain in AP is acute in nature while pain in CP is chronic. CP symptoms can include abdominal pain radiating to the back, worsening of pain after eating, especially after fatty meals, exocrine and endocrine insufficiency, and the presence of anxiety (Walsh et al., 2012, Poulsen et al., 2013).

CP is associated with irreversible damage to the pancreas as well as the presence of fibrosis (Poulsen et al., 2013). Pancreatic tissue becomes fibrotic reducing function. Eventually blood sugar regulation and food digestion are affected. CP pain remains a possibility at any time for someone with CP. The pain of CP increases when neuropathy is present (Poulsen et al., 2013). Neuropathy includes sprouting of the intrapancreatic nerves, and activation of immune cells (Poulsen et al., 2013).

Nociceptive stimuli are sensed by a variety of nerve cells that can detect nociceptive stimuli or injury and transduce them into electric signals (Pasricha, 2012, Poulsen et al., 2013). Primary afferent nerves have their cell bodies in the dorsal root ganglia (DRG) next to the spinal cord (Poulsen et al., 2013). These nociceptors begin as nerve endings in a particular tissue and insert their central endings into the dorsal horn of the spinal cord. The peripheral nerve receptors

detect physical or chemical stimuli. An action potential can move through the nerve to the dorsal horn of the spinal cord. Neurotransmitters are released and secondary transmission neuron axons carry the pain signals to the brain. The sensation of pain is formed in humans from this stimulus.

Prolonged peripheral nerve stimulation, for example from a continuing injury, can lead to the sensitization of nociceptors by neurotransmitters. Nociceptor plasticity of this kind can result in a reduction in activation threshold, an increased response to pain stimuli, and can give the impression of spontaneous activity (Basbaum et al., 2009, Poulsen et al., 2013). Sensitization of peripheral nerves in this manner is called peripheral sensitization.

Peripheral sensitization can also sensitize central pain transmitting neurons in the dorsal horn (Basbaum et al., 2009, Poulsen et al., 2013). Central sensitization can accompany strong and/or continuous noxious peripheral stimuli, and tissue or nerve damage (Poulsen et al., 2013). The overarching effect of central sensitization is felt as pain in humans. This pain no longer reflects the actual presence, intensity, or duration of pain emanating from the periphery (Poulsen et al., 2013).

CP patients show increases in neurotransmitters associated with pain sensation such as calcitonin gene regulatory product (CGRP), Substance P (SP), nerve growth factor (NGF), brain-derived neurotropic factor (BDNF), and proinflammatory cytokines (Basbaum et al., 2009, Poulsen et al., 2013). Certain neurotransmitters activate the nerves innervating the inflamed pancreas and cause excitation of the secondary transmission neurons. Antagonizing each of these neurotransmitters pharmacologically decreased pain in rats with chronic pain (Basbaum et al., 2009).

Afferent nerves carry nociceptive signals to the central nervous system following the detection of stimuli from nociceptors. One such nociceptor neuron channel of particular interest

due to its contribution to pain in CP is the transient receptor potential cation channel subfamily V member 4 (TRPV4). The TRPV4 channel senses mechanical pressure, as well as heat, and is activated by alcohol metabolites (Ceppa et al., 2010, Everaerts et al., 2010).

One theory as to the development of CP is that mechanical pressure can increase in the damaged pancreas due to clogged ducts as alcohol decreases the solubility of proteins in the ductal space and increases fibrosis, leading to plugging of the ducts and the subsequent autodigestion of the pancreas (Masamune et al., 2009, Braganza et al., 2011). The literature shows no agreed upon correlation between "general" pancreatic fibrosis and the amount of mechanical pressure due to plugged ducts. Fibrosis does not show a specific development or progression through a specific area in the pancreas such as the head, body, or tail. However, the Necrosis/Fibrosis hypothesis asserts that focal areas of damage caused by insult to the pancreas become necrotic and then are replaced with fibrosis (Witt et al., 2007).

The nerves of the mouse pancreas associate with blood vessels of different sizes. Post-ganglionic sympathetic nerve fibers, for example, are associated with the acini and capillaries (Lindsay et al., 2006). The peri-vascular mesenteric nerve plexus associates along the superior mesenteric artery as they pass through the pancreas (Lindsay et al., 2006). The largest blood vessels were found in association with A-delta and A-beta fibers (Lawson and Waddell, 1991, Lindsay et al., 2006). CGRP positive sensory fibers enter the pancreas by the splanchnic nerve through the mesenteric and celiac ganglion in the rat (Su et al., 1987). CGRP-positive small unmyelinated and medium diameter thinly myelinated sensory nerve fibers are seen in association with large and medium sized blood vessels as well as the islets (Lindsay et al., 2006).

Lindsay et. al (2006) found in the mouse that sensory nerves containing CGRP are characterized as A-delta or C-fibers. CGRP-positive sensory fibers (A-delta or C-fibers) are

located at highest densities in the pancreatic head, next highest densities in the body, then the lowest density in the tail of the pancreas (Lindsay et al., 2006). Examples of nociceptive receptors found on A-delta or C-fibers include bradykinin receptors (Ma, 2001), tyrosine kinase A (TrkA) receptors (Averill et al., 1995), transient receptor potential vanilloid-1 (Michael and Priestley, 1999, Hwang et al., 2005), proteinase-activated receptor 2 (Hoogerwerf et al., 2001), transient receptor potential vanilloid-4 (Zhang et al., 2015). Nociceptors such as these mediate acute pancreatic pain (Hoogerwerf et al., 2001, Wang and Westlund, 2001, Winston et al., 2003, Hoogerwerf et al., 2004).

As a visceral organ the pancreas displays little pain signaling under non-pathological conditions. Damage to the pancreas results in pain signaling. The TRPV4 channel located in the pancreatic nerves (Ceppa et al., 2010) is becoming well known as a source of pain signaling in pathological conditions. Damage to pancreatic parenchyma by alcohol and fat has been shown to increase the TRPV4 in the pancreas (Zhang et al., 2013, Zhang et al., 2015) in a rat AHF model. Pancreatic afferent nerves connect to second order neurons in the spinal cord that are sensitized by the continuous presence of alcohol and other substances (Forsmark, 2013). Increased TRPV4 is thought to increase pain signaling in human CP patients.

Alcohol and fat contribute to damage and nociceptive signaling of the pancreas. For example, Zhang (2013) demonstrated that the pancreatic stellate cells isolated from the pancreas of rats fed the AHF diet are activated by alcohol. Increased numbers of TRPV4 are the likely cause for increased pain signaling as they are activated by alcohol and fat (Ceppa et al., 2010, Zhang et al., 2013, Zhang et al., 2015). TRPV1 and P2X7 channels are also activated in pancreatitis (Kunzli et al., 2007, Ceppa et al., 2010, Schwartz et al., 2013). Alcohol damages the pancreas, activating neurogenic inflammation (NI), and causing release of Substance P (SP)

(Ceppa et al., 2010). SP causes the release of proinflammatory mediators to occur (Vera-Portocarrero and Westlund, 2005).

As damaging alcohol-related conditions in the pancreas continue, fibrosis and tissue atrophy within the pancreas increases (Forsmark, 2013). The pancreas processes ethanol with fatty acids (FA) from high fat foods, to produce fatty acid ethyl esters (FAEE) (Siech et al., 2009). FAEE, as well as reactive oxygen species (ROS) produced from the ethanol, damage the pancreatic cells. The pancreatic stellate cells eventually form fibrosis that interferes with pancreatic functions (Masamune et al., 2009) as they attempt to repair damaged pancreatic cells (Siech et al., 2009).

Pancreatic histopathology of mice fed the alcohol and high fat diet showed morphological disruption common to CP: atrophy, fibrosis, diffuse nuclear material and poor cellular architecture. Fibrosis interferes with and eventually halts the functions of the pancreas. Cellular architecture refers to the orderly arrangement of acinar cells into an acinus with duct. As pancreatic functions slow, comorbidities such as diabetes mellitus type 3c and poor nutrition occur (Cui and Andersen, 2011, Ewald and Bretzel, 2013).

Loss of exocrine function can cause a specific form of diabetes called diabetes mellitus type 3c (T3cDM). T3cDM refers to loss of function in the endocrine pancreas due to pancreatic damage caused by either acute or chronic pancreatitis of any type (Ewald and Bretzel, 2013). Diagnosis of T3cDM is hampered by the prevalence of type 2 diabetes mellitus (T2DM) which is prevalent in the population at 8% (Gudipaty, 2015). A study by Ewald showed that 10% of patients at a German academic clinic had T3cDM, but had been classified as T2DM (Ewald et al., 2012). Diagnostic criteria for T3cDM include but are not limited to: exocrine pancreatic

insufficiency, positive pancreatitis imaging, and the absence of type 1 diabetes mellitus (Ewald and Bretzel, 2013).

4.4.8 The Epidemiology of Alcoholism

The development of alcoholic CP is impacted by the genetics and behaviors of those who are doing the drinking, how much they drink, as well as their pattern of drinking. Just under 65% of American adults drink alcohol (NCHS, 2013). 30% of people who drink in America are heavy drinkers (NIAAA, 2010). See next paragraph for the definition of light, moderate and heavy drinking categories. Hasin et al. (2007) determined that 17% of men and 8% of women will at some time in their lives meet the criteria for alcohol abuse. Drinking, when begun at an early age, can lead to the development of alcohol dependence (Hingson, 2009). The number of current drinkers in the American population peaks between 25-44 years, with a steady decline as age increases (NCHS, 2013).

The pattern of drinking adopted is used to determine whether someone is a heavy, moderate, or light drinker (NIAAA, 2010). Light drinkers drink less than 3 drinks a week on average (NIAAA, 2010). Low risk drinking levels are understood as ≤4 drinks a day and/or ≤ 14 drinks a week for men, and ≤3 drinks a day and/or ≤7 drinks a week for women (NIAAA, 2010) and those over 65 (NIA, 2011). High risk drinkers consume greater than 4, or 3 drinks a day and/or may have greater than 14, or seven drinks a week (depending on sex) (NIAAA, 2010). “Binge drinking”, a process of drinking greater than 5 drinks in one day, peaks at between 18-24 years, and then falls steadily to age 64 (NCHS, 2013). A steep drop in binge drinking occurs at 65 years, and also at 75 years and older (NCHS, 2013). Binge drinking is a particular risk for pancreatitis (Irving et al., 2009). Heavy drinking that occurs at younger ages damages the pancreas, decreasing physiological reserve, but may not impact perceived health until later in life.

Blood alcohol concentration (BAC) is a measure of alcohol concentration in the blood. The BAC changes depending on how much and what type of alcohol has been consumed over a period of time (Cox et al., 1985, Fisher et al., 1987). At the low end of the range (BAC=0.021) humans may exhibit slight impairment (NHTSA, 2005). A BAC of 0.08 in a human is considered legally intoxicated (NHTSA, 2005). For example, a BAC of 0.086 would result in a loss of balance and reaction time in humans (NHTSA, 2005). Please see page 89 for more information.

4.4.9 The Epidemiology of a High Fat Diet

Rats fed the AHF diet develop chronic pancreatitis (Zhang et al., 2014, McIlwrath and Westlund, 2015, Zhang et al., 2015). The particular type of fat needed to induce pancreatitis, beyond that used in the literature, is beyond the scope of this dissertation. Cardiovascular complications due to obesity from the high fat diet were neither quantitatively nor qualitatively observed, nor were they seen in mice used in the research. One reason why the “high fat” part of the AHF diet is justified for use in mouse models of CP is due to rats reliably developing CP on the AHF diet. Another reason for using the “high fat” part of the AHF diet is that obese humans are at increased risk of developing CP (Sadr-Azodi et al., 2013). A consideration for the use of the “high fat” part of the diet is that AP can be brought on by high fat meals (Thomas et al., 2012). Recall that AP can progress to CP, especially in the presence of alcohol (Thomas et al., 2012).

Obesity (BMI >30) (NCHS, 2013) is likely to occur upon consumption of a high fat diet. Americans on average, consume more calories than necessary. The American diet has increased per capita fat consumption from 120 g in 1909 to 170 g in 2000 (Gerrior, 2004). This rise in per capita fat consumption (Gerrior, 2004) is a likely contributor to the current obesity epidemic.

Excess calories can be consumed in fast food, foods consumed during sports events, at celebrations, and on holidays, among others. Sugared drinks including soda and coffee contain a "hidden" source of calories as people may not realize and track the number of calories present in their drinks. 50% of Americans consume at least one sugared drink per day (Ogden, 2011, Harvard, 2012).

One possible reason for the overconsumption of calories in any form is a lack of knowledge or understanding of portion sizes. Nutrition labels are present on consumables and inform on a number of variables including calories per serving, serving size, as well as amount of sugar, protein, and other characteristics of the consumable (CDC, 2015b). Excess calories are converted to fat by the body, contributing to obesity.

Obesity doubles the risk of developing CP (Sadr-Azodi et al., 2013) and can occur due to consuming more calories than are expended. More than 27% of Americans are currently obese; nearly 35% of non-obese Americans are overweight (BMI 25-30) (NCHS, 2013). The percent of obese Americans increases in a stepwise fashion from about 17% at 18 to 32% at 64 years (NCHS, 2013). Thereafter obesity declines from about 31% between 65-74 years and to a low of about 18% in those older than 74 years (NCHS, 2013).

4.4.10 Anxiety and Pancreatitis Histology

The association of pancreatitis histology with anxiety has been understudied. Anxiety, like pain, is useful as a transient state but when anxiety is prolonged it is termed a disorder. Anxiety can cause a mouse to pay better attention to its surroundings so it can avoid a possible predator. As a transient state, anxiety allowing escape from a predator is useful. Long-term increased anxiety, however, could interfere with daily survival, for example, a mouse regularly too anxious to eat a full meal due to continually checking for predators. Steimer (2002)

suggested that anxiety is defined as “a psychological, physiological, and behavioral state induced in animals and humans by a threat to well-being or survival, either actual or potential.” Overarching anxiety of this kind in humans is characterized by “a marked, persistent, & excessive or unreasonable fear” (DSM-4) that interferes with normal life (Roy-Byrne et al., 2008). Anxiety is often comorbid with pain and depression (Kroenke et al., 2013). Therefore, it is important to determine anxiety in mice with CP histology.

4.4.11 Summary

Etiologically relevant animal models for study of CP are needed. In order to begin filling this gap a central purpose of this dissertation research was to examine relationships between the AHF diet and pancreatitis with attention to hypersensitivity and anxiety-like behaviors. The alcohol and high fat diet induced pancreatitis described here mimics human etiological risk factors of alcohol and high fat consumption for advancement to alcoholic chronic pancreatitis. This model was characterized for the interaction of the chronic pathological state, persisting pain-, anxiety-, and fear-like behaviors.

Pancreatitis is painful, and decreasing pain in this patient population is a priority. Current medications for the chronic pain of chronic pancreatitis do not relieve the pain long-term. Initially the pain can be relieved with opiates. Effective opiate doses for pain control must increase and increase (Nusrat et al., 2012, Raffa and Pergolizzi, 2014). Eventually the patient becomes resistant to opiates. The alcohol and high fat chronic pancreatitis mouse model will be a tool for the research of chronic pancreatitis and possible medications that could for use by people with CP.

5 SIGNIFICANCE OF RESEARCH

The AHF diet is generally similar, though not a perfect match, of the overconsumption of alcohol as well as a high calorie diet seen etiologically in humans that develop alcoholic pancreatitis (Irving et al., 2009, Yadav and Lowenfels, 2013). While humans eat a wide variety of diets, the laboratory environment required a homogenous diet of either Teklad 8626 (Teklad #8626, Harlan, Indianapolis, IN), or TestDiet LD101A (TestDiet LD101A, Richmond, IN). Teklad 8626 is a standard chow fed to mice in scientific environments. The first eight ingredients of Teklad 8626 pellets are ground wheat, fish meal, dried skimmed milk, porcine fat, soybean oil, casein, wheat middlings, and brewers dried yeast. The first eight ingredients of the liquid AHF TestDiet 101A mix are vitamin-free casein, olive oil, maltodextrin, dried corn syrup, soy fiber, corn oil, suspension colloid, and safflower oil. Please see Appendix B and C for the nutritional analysis. Laboratory conditions for mice are different than those faced by humans. However, consumption of the AHF diet does mimic two factors, overconsumption of alcohol seen in alcoholic chronic pancreatitis, as well as a high fat diet.

Diets high in fat and alcohol increase exposure to etiological risk factors for pancreatitis. Takano et al. (1992) showed that stress, which can both cause and be caused by anxiety (Steimer, 2011), can aggravate CP progression. Production of this nontransgenic mouse model of AHF-induced CP without the use of injected chemicals, provides a model more closely focused on the etiology of human pancreatitis In addition, the production of this model in mice allows for the future use of transgenic mice to further the study of alcoholic pancreatitis and associated conditions. The AHF CP mouse model also allows for study of the effects of anxiety on pancreatitis histology.

6 RESEARCH STUDY DESIGN

6.1 Study Model

The mice used in the study were floxed TGFβ1$^{flox\text{-}ex6}$ on a mixed C57BL/6 background, from a strain maintained at The Jackson Laboratory. These mice were without the ability to express Cre recombinase. Without Cre recombinase floxed TGFβ1 cannot be removed using the cre-lox system. The mice were in a pre-knock-in/knock-out condition, so are otherwise normal.

This paragraph highlights the Lieber & DeCarli (1989) diet which is used with varying success by many researchers, and illustrates how the mice were fed alcohol on a certain schedule, as well as dietary high fat. Mice were fed a liquid diet including up to 6% alcohol. The first week the mice were given the liquid diet with 0% alcohol, then they were given 4%, then 5%, and finally they were maintained at 6% for the remainder of the research. The diet is composed of 57% fat contributed by corn oil in the liquid diet as well as supplemental lard. This liquid diet is a modified Lieber & DeCarli (1989) diet used with great success in our lab to induce CP in rats (Yang et al., 2008, Zhang et al., 2014, McIlwrath and Westlund, 2015), confirmed using histology of the whole pancreas following sacrifice of the rats, as well as studies of nociceptive behavior. The success of the diet in rats, and the genetic similarity of mice to rats, allowed the expected result to be chronic pancreatitis. The presence of CP in this new mouse model was confirmed by analyzing pancreatic tissue for focal fibrosis, atrophy, and degradation of acini ducts.

The production of this mouse model allowed study of anxiety- and fear-like behaviors, as well as CP in older mice. Typical mice used for experimentation are 8 weeks old. This study began with mice that were 4.5 months, or 18 weeks old. The age range in mice, from 4.5 to 9 months old, spans about 20 human years and relates to adult human maturity proceeding from the early twenties to about forty years (Jackson Laboratory, 2011). The study timeline continued

for 19 weeks. Studying the model in older mice is important as the incidence of acute pancreatitis, often a precursor to CP, increases with age (Nusrat et al., 2012, Yadav and Lowenfels, 2013).

6.2 Specific Aims and Study Design

6.2.1 **Specific Aim 1 Study Design: To support the hypothesis that mice fed a liquid alcohol and high fat (AHF) diet will develop hypersensitivity and sustain damage to the pancreas seen in chronic pancreatitis.** The hypersensitivity and pancreatic damage seen in the AHF mouse model are consistent with that commonly seen in the AHF rat model of CP, namely the presence of pain-like behaviors, and markers for pancreatic damage such as fibrosis, fatty infiltration, and atrophy (see Table 4 below).

Table 4	Mouse Groups: Control and AHF Number: n=6 (control), n=6 (AHF)
Establish Hypersensitivity and Pancreatitis Histology	
Hypersensitivity ❖ von Frey ❖ Modified Hotplate ❖ Smooth or Rough Mechanical Plate	Pancreatitis Histology ❖ Fibrosis ❖ Acinar Atrophy ❖ Ductal Degradation ❖ Steatosis (fatty infiltration)

Mice in the AHF group were fed the AHF diet for the duration of the study as described previously. The presence of pancreatitis was verified using pain-like behavior and tissue samples.

A pharmacological study designed to alleviate CP-induced hypersensitivity by antagonizing the TRPV4 channel (HC067047) was conducted as shown in Figure 3 and in 6.3 Test Drugs**.** The experiment was designed to test whether antagonizing TRPV4 would decrease hypersensitivity

in AHF mice. If the mice developed CP, then antagonizing TRPV4 should decrease nociceptive-related behaviors. If the mice had not developed CP then antagonizing TRPV4 should have no effect. Hypothesis: Mice with AHF-induced CP will show decreased nociceptive behaviors upon dosing with the TRPV4 antagonist.

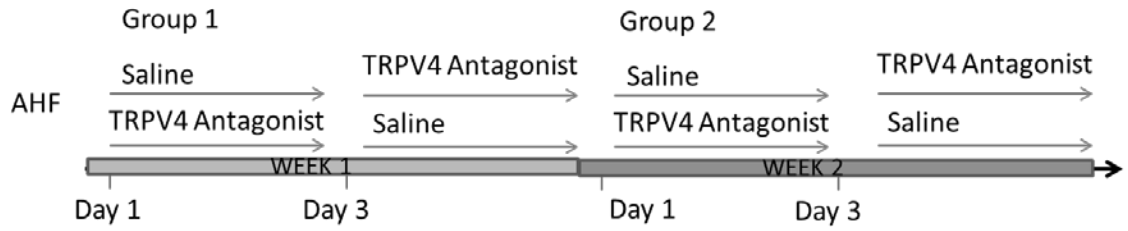

Figure 3. Experimental Design of TRPV4 Antagonist H067047 Testing. Experimental design to test that antagonizing TRPV4 decreased hypersensitivity in AHF mice nociceptive-related behaviors.

Relevance to Aim 1. Hypersensitivity present in the AHF mice decreased with the addition of the TRPV4 antagonist. TRPV4 is present and active in the signaling of hypersensitivity in rats with pancreatitis (Ceppa et al., 2010). HC067047 decreased TRPV4 signaling in the AHF mouse model. This agrees with data from our lab indicating that HC067047 decreased signaling of TRPV4 in rats with alcohol and high fat induced chronic pancreatitis (Zhang et al., 2015).

A mu-opioid receptor agonist, Loperamide HCL, was used next and was expected to increase endogenous opioid receptor signaling known to be present on the pancreatic nerves and thus decrease hypersensitive behavioral responses. Loperamide was applied as described below in Figure 4 and in section 6.3 Test Drugs.

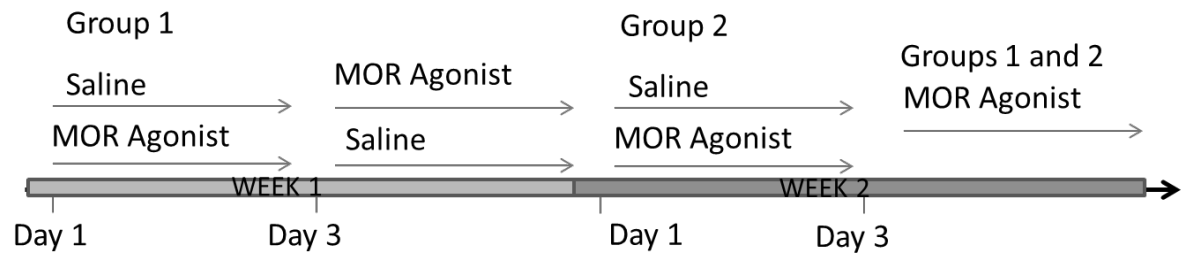

Figure 4. **Experimental Design of mu-Opioid Agonist Loperamide HCl Testing.** Experimental design to test that activating mu-opioid receptors decreased hypersensitivity in AHF mice.

6.2.2 **<u>Specific Aim 2 Study Design</u>: To characterize anxiety- and fear-like behaviors in mice using non-reflexive tests. Hypothesis: Mice on the AHF diet will display higher anxiety- and fear-like behaviors than control mice in non-reflexive testing.** While AHF-fed and control rats in other studies responded in a quantifiable manner to testing, such as the hotplate, mice are historically less reliable than rats using reflexive testing. For example, in the hotplate test mice showed very few differences in the typical threshold markers seen in rats such as licking the hind paw. Mouse testing methods returning reliable results were developed using non-reflexive methods to optimize behavioral testing in this AHF CP mouse model. The following assays were used to measure mouse anxiety- and fear-like behaviors: plus maze, open field, place preference and escape, counts of grooming, counts of rearing, and number of uncued freeze events. Non-reflexive testing allows for a more holistic view of the mouse study treatment groups, highlighting anxiety-like and fear-like behaviors present in the animal. Note that anxiety interacts with pain reported in the human clinical patient (Walsh et al., 2012, Bair et al., 2013), therefore, it is necessary to include anxiety testing in this mouse model of human disease for possible future research into the possibility of an anxiety/nociception interaction. While anxiety- and fear-like behaviors are not expected to be directly translatable to humans this is an understudied area of CP model research well suited for study in this model.

Table 5	Mouse Groups: Control and AHF Number: n=6 (control), n=6 (AHF)
Non-reflexive Testing in a Chronic Pancreatitis Mouse Model	
	Week
1. Dark/Light Place Preference	1 - 9
2. Smooth/Rough Place Preference	1 - 8
3. 44°C/22°C Place Preference	3 - 10
4. 44°C/22°C Escape	3 - 10
5. Reflexive testing	7 - 19

Table 6	Mouse Groups: Control and AHF Number: n=6 (control), n=6 (AHF)
Anxiety and Pancreatitis Histology	
Quantitative	Pancreatitis Histology
❖ Freezing	❖ Fibrosis
❖ Grooming	❖ Acinar Atrophy
❖ Transitions	❖ Ductal Degradation
❖ Plus Maze	❖ Steatosis (fatty infiltration)
❖ Open Field	

6.3 Test Drugs

6.3.1 TRPV4 antagonist HC067047

HC067047 is a potent and selective inhibitor of the transient receptor potential family cation channel 4 (TRPV4) (Everaerts et al., 2010). TRPV4 is found on nerves in the pancreas, and senses osmotic pressure as well as metabolites of alcohol (Ceppa et al., 2010). TRPV4 increases in CP and serves a role in neurogenic inflammation and pain (Ceppa et al., 2010). Everaerts (2010) has shown that this antagonist inhibits the TRPV4 mediated response to heat and arachadonic acid. The antagonist is efficacious at 10 mg/kg when given intraperitoneally (i.p.).

HC067047 (2-Methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-*N*-[3-(trifluoromethyl) phenyl] -1*H*-pyrrole-3-carboxamide) was obtained from TOCRIS (San Diego, CA. The efficacy of this drug for reduction of pain- and anxiety-like behaviors was tested in mice with CP. Injections were made intraperitoneally (i.p.) at 10 mg/kg, after which the mouse was returned to the home cage for at least 30 minutes. Testing began at 60 minutes after injection. This time frame was calculated as a time of high effectiveness using the HC067047 dose response curve generated by the authors of the Everaerts (2010) paper. Prolonged alcohol and high fat exposure has been shown to increase TRPV4 in pancreatic innate immune stellate cells in another study in our lab (Zhang et al., 2013).

6.3.2 Mu-opioid Receptor Agonist Loperamide

Loperamide HCL (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-N,N-dimethyl-2,2-diphenylbutanamide) is a peripherally restricted, mu-opioid receptor (MOR) agonist commonly used to treat diarrhea (Awouters et al., 1993). Loperamide was used to test the effects on behavioral measures of reducing peripheral nerve activation in mice with CP. Loperamide was suspended in a 20% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Milwaukee, WI) in saline and diluted to the proper dose concentration. Loperamide injections were made i.p. at 0.4, 0.6, 0.8, or 1.2 mg/kg and testing began at 60 minutes after injection (Reichert et al., 2001, Everhart and Ruhl, 2009).

7 Statistical Analysis

T-testing and repeated measures one and two-way ANOVA were used to analyze the results of testing. p values at less than 0.05 were significant.

8 ANXIETY AND ALCOHOL: RELEVANCE TO FUTURE STUDIES OF THE INTERACTION OF ANXIETY AND ALCOHOL

8.1 Anxiety, Stress, and Disease

8.1.1 Adaptation to Chronic Stress

Anxiety is a manifestation of stress. Chronic stress decreases organ health due to interactions with stress adaptation in the body (Dallman et al., 2003). Mice with more stress/anxiety behavioral affect are likely to have decreased pancreatic health. The hypothesis tested is that AHF mice with visceral pain-like behaviors will display greater anxiety-like behaviors than the control group.

8.1.2 Alcohol Abuse and Disease

In humans, stressful events produce anxiety, which can increase harmful behaviors such as alcohol abuse (Kushner et al., 2005). An increase or maintenance of heavy drinking over a period of time has been shown to increase the chance of damaging the pancreas irreversibly, assisting in the development of CP (Schneider et al., 2012, Zechner et al., 2012). The risk of developing pancreatitis increases in an alcohol dose-dependent manner (Irving et al., 2009).

8.1.3 Mouse Behaviors and Human Anxious Affect

Affect has been shown to influence human reaction to pain stimulus, increasing severity of pain perceived (Bair et al., 2013). It has been demonstrated that counseling to relieve feelings of anxiety and manage stress decreases healthcare use (Madan et al., 2013) and therefore is likely to impact compliance in the case of patients with CP. Different mouse strains demonstrate different levels of behaviors equated with anxious/stressed affect in humans. Mice in the present research had nesting materials available, but additional materials such as paper tubes could be supplied. In future studies, because AHF mice demonstrate high anxiety-like behaviors, and disordered histology, the next step in continuing studies would be to determine if low anxiety

behavior mice show the same severity of pancreatitis histology. Gurfein et al. (2012) alleviated stress in mice by providing nesting material, a play tube, and a den-like home in the cage. Additions like these that are meant to decrease anxiety in mice could help to slow the progression of CP. Calming human stress/anxiety could slow the progression of CP in humans.

8.1.4 Anxiety and Disease

Nineteen million US adults experience anxiety disorders. The stress-depression-anxiety axis has been shown to contribute to the development of diseases such as CVD, HIV, and cancer (Krantz and McCeney, 2002, Leserman et al., 2002, Roy-Byrne et al., 2008). In light of the literature showing the acceleration of disease in the presence of psychological stressors, it is possible that an accelerated progression to CP through increased alcohol consumption, smoking, and higher fat content in the diet may occur. Future studies will determine the part anxiety plays in the observed histology of CP in the AHF-induced pancreatitis seen in this model. This dissertation research provides a new etiologically based model of CP, making study of the disease in mice more etiologically relevant to the disease seen in humans.

9 INTRODUCTION TO CHAPTERS

This dissertation is comprised of four chapters within the context of development of a new mouse model of diet-induced CP, and assessing anxiety- and fear-like behaviors present in control or treated animals in this new model. **Chapter one** consists of an introduction to the background and literature. Chronic physical and mental pain increase vulnerability to future threats, leading to increased mortality at a younger age. Chronic pain tends to increase as a person ages. People in chronic pain experience physical, social, and mental decreases in function (Figure 3) (Fine, 2011). Pain in this sense encompasses physical pain such as that of CP, as well as psychological pain manifestations such as anxiety.

In **chapter two**, the specific aim was to produce a wild type mouse model of CP induced only by the consumption of an alcohol and high fat (AHF) diet. This diet has produced CP in rats. The production of a mouse model of CP is expected to pay dividends in decreased general costs of maintaining and feeding the mice, while allowing future studies on transgenic mice with AHF induced CP. Mice fed the AHF diet over a period of five months did demonstrate pain related behaviors, as well as histology indicative of CP. This supports the presence of face validity, which is defined by Campos as the presence of physical and behavioral characteristics of the disease (Campos et al., 2013). It is suggested that the impact of both alcohol and high fat in a non-surgical, nontransgenic mouse model of CP, better recapitulates etiological factors known or suspected of leading to the development of CP in human patients in clinic, and provides construct validity. Construct validity is shown by following the known etiology of the disease in models of the disease. The production of this novel "diet alone" induced CP mouse model answers the call of experts in the field of pancreatitis for a model with alcohol as an induction factor. Note that alcohol alone is unsuccessful in CP model induction (Reed, 2014).

Chapter three is an investigation into the presence of anxiety- and fear-like behaviors in AHF induced CP mice. Traditional methods of testing can return unreliable results in the mouse, in addition, non-reflexive methods require less training to learn and score, releasing highly skilled laboratory personnel to complete more complex testing. Mice fed the AHF diet showed significant behavioral readouts in non-reflexive testing paradigms in comparison with control mice. It is proposed that non-reflexive testing is a suitable method of behavioral testing in a mouse visceral pain model. Exploration of the AHF CP mouse model is important to determine relevance to the human pain-cycle of anxiety, depression, and pain. Modification of possible anxiety-inducing factors in humans at-risk for CP may produce a decrease in progression of

recurrent acute pancreatitis to CP; in the context of the mouse model, further testing would include enrichment of the home cage with nesting material and a dark hiding place to determine the effect on disease presence.

Chapter four provides a summary, conclusions, and future work. Physiologic reserve tends to decrease as the body ages. Chronic physical and mental pain are symptoms of vulnerability and decreased homeodynamic space, leading to increased mortality at a younger age. Maintenance of high physiologic reserve is diminished in populations such as alcoholic chronic pancreatitis patients due to overconsumption of alcohol, and consumption of high fat foods. Alcohol and high fat foods can be comforting and assist in an individual's control of stress in their life (Gilman et al., 2008, Tomiyama et al., 2011)

One way to help add more years to life and more life to years is to understand why disease affects people in populations heterogeneously, and study how to relieve the symptoms of painful diseases such as CP. The study of CP in a non-surgical mouse model is essential to understand the disease. The AHF diet induces the morbidity of CP in mice likely in an etiologically similar manner to that in humans. The research data presented here, as well as literature reviews on the subject, highlight the damage caused to the pancreas with continued overconsumption of alcohol in the presence of a high fat diet.

CHAPTER TWO

A Mouse Model of Chronic Pancreatitis Induced by an Alcohol and High Fat Diet

ABSTRACT

Background/Aims: While study of acute pancreatitis in chemically-induced rodent models has provided useful data, models of alcoholic chronic pancreatitis induced only by diet have not been available in mice. The aim of the present study was to characterize a mouse model of CP for laboratory study induced with an alcohol and high fat (AHF) diet.

Methods: Mice were fed normal chow ad libitum or a liquid high fat diet containing 6% alcohol as well as a high fat supplement (57% total dietary fat) over a period of five months. Hind paws of the mice were tested for mechanical hypersensitivity with von Frey filaments and the mechanical plate. A modified hotplate testing procedure determined higher order responses to visceral hypersensitivity. Mice underwent mechanical and thermal testing both with and without pharmacological treatment. Pharmacological agents tested were a peripherally restricted μ-opioid receptor agonist, Loperamide or a TRPV4 antagonist, HC067047.

Results: Mice on the AHF diet exhibited mechanical and heat hypersensitivity as well as histopathology indicative of chronic pancreatitis. Loperamide attenuated both mechanical and heat hypersensitivity, while heat hypersensitivity decreased after TRPV4 antagonism.

Conclusion: Mice fed an alcohol and high fat diet develop histopathology consistent with chronic pancreatitis, as well as opioid sensitive mechanical and heat hypersensitivity.

1. INTRODUCTION

In 2004, pancreatitis cost the US $373.3 million in direct and indirect costs (Everhart and Ruhl, 2009). Eight in 100,000 people are diagnosed with CP in the US yearly (Yadav and Lowenfels, 2013) and up to 50% of these can live 20 years after diagnosis with CP (Braganza et al., 2011). Fifty out of 100,000 people are living with CP (Yadav et al., 2011b, Hirota et al., 2012). Many of these patients endure severe intractable pain.

The development of CP in humans results from multiple possible contributing risk factors, one of which, described by Forsmark (2013) is the sensitizing effect of alcohol on the pancreatic cells. Overproduction of extracellular matrix by alcohol-activated pancreatic stellate cells eventually promotes the fibrosis that interferes with pancreatic functions (Masamune et al., 2009). This overproduction occurs as pancreatic stellate cells attempt to repair damaged pancreatic cells (Siech et al., 2009). It has been suggested that high levels of alcohol also decrease the solubility of proteins in the ductal space, leading to plugging of the ducts and the subsequent autodigestion of the pancreas (Braganza et al., 2011).

The National Commission on Digestive Diseases (NCDD) under the umbrella of the National Institute of Diabetes and Digestion and Kidney Diseases (NIDDK), value the production of better animal models to further the study of alcoholic pancreatitis (Witt et al., 2007). Mouse models in which CP is induced only with a combination of ad libitum high fat liquid food with added alcohol and lard supplementation do not currently exist. Current rodent models of pancreatitis induced by chemical irritants such as cerulein or dibutyltin dichloride (DBTC) have proven fruitful for studies of acute pancreatitis (Perides et al., 2005). However, these models do not provide the range of symptoms for CP based on risk factors seen in the clinic.

The alcohol and high fat (AHF) diet proposed for use in this model is a modified Lieber-DeCarli (Lieber and DeCarli, 1989) diet containing 6% ethanol, added corn oil (20% fat) and supplemental lard. This liquid diet has been used with great success in rats to induce CP, confirmed using histopathology of the pancreas and pain-related behavioral studies (Yang et al., 2008, Zhang et al., 2014, McIlwrath and Westlund, 2015, Zhang et al., 2015). The purpose of the present study is to produce an AHF diet-induced CP mouse model, characterize pancreatic pathology, and also the pain-related behaviors which develop in mice fed the alcohol and high fat diet. Histopathological confirmation of pancreatitis includes evidence of significant pancreatic fibrosis, as well as diffuse nuclear material, and poor cellular architecture.

2. METHODS

The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.1 Induction of Pancreatitis

Five month old male mice on a C57BL/6 background weighing less than 40g (The Jackson Laboratory) were used for this study. Animals were housed at 21-24°C, on a reverse light:dark 14:10 hour schedule. Control mice were fed rodent chow (Mouse Breeder Diet 8626, 10.4% fat) from Teklad (Madison, WI). AHF pancreatitis mice were fed liquid diet from Test Diet (LD101A, Richmond, IN) and supplementary fat (lard and corn oil). The total fat content was ~57%. Alcohol was gradually added to the diet over a period of weeks from zero, to 4%, 5%, and then 6%. AHF fed mice were maintained at 6% alcohol for the remainder of the study. Body weight was monitored weekly.

2.2 Assessment of Pain Related Behaviors

2.2.1 Paw Withdrawal Threshold (PWT) Testing

Mice were loaded into Plexiglas rectangles (7x4x4 cm) on a raised wire-bottomed table and allowed to acclimate for 30 minutes. Von Frey filaments were applied in a methodical manner to the plantar surface of the mouse hind paws as described in Chaplan et al. (1994) to assay mechanical thresholds. The von Frey monofilaments ((4.74) 6.0g; (4.31) 2.0g; (4.08) 1.0g; (3.61) 0.4g; (3.22) 0.16g; (2.83)0.07g; (2.36) 0.02g; (1.65) 0.008g)) were applied perpendicular to the plantar surface until just bent and held for 5 seconds. A positive response was scored when the paw withdrawal response was observed (animal picked up its foot, flicked it) away from the monofilament either during the time the filament was pressed against the plantar surface or just after it was removed. The up-down method suggests that when a positive response is scored, the next lower filament is used, while if there is no response (negative response), the next higher filament is used. Testing continued until an additional four filaments returned a positive or negative response. The up-down algorithm was then used to calculate the 50% paw withdrawal mechanical threshold (in gram force) (Chaplan et al., 1994). The decreased paw withdrawal threshold is an indication of mechanical allodynia.

2.2.2 Smooth or Rough Mechanical Plate

Rearing exploratory activity, including frequency and duration, with or without a rough textured floor insert (the textured side of a polystyrene ceiling light panel diffuser (89091), Lowes.com), was monitored in real time and captured using custom software during 5 min tests. The test apparatus consisted of a clear Plexiglas box (11cm X 8cm X 8cm) placed upon either a smooth or roughly textured surface.

2.2.3 Modified 44°C Hotplate Assay

The procedure is as in Kline and Wiley (2008), and will be described here. In modified 44°C hotplate testing, mice were first placed on a 38°C hotplate for ten minutes in order to slowly pre-warm the animal's foot. Thereafter the mice were moved to a 44°C hotplate for a ten-minute testing period (Kline and Wiley, 2008). In this study, jumping events, rearing events, and latency to first jump were the dependent variables and were recorded using custom software.

2.3 Test Drugs

2.3.1 Mu-opioid Receptor Agonist

Loperamide HCl (SIGMA-Aldrich, Milwaukee, WI) is a peripherally restricted, mu-opioid receptor (MOR) agonist commonly used to treat diarrhea (Awouters et al., 1993). Loperamide HCl was suspended in a 20% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Milwaukee, WI) in saline and diluted to the proper dose concentration. Loperamide HCl injections were made intraperitoneally (i.p.) at 0.4, 0.6, 0.8, or 1.2 mg/kg and testing began at 60 minutes after injection (Reichert et al., 2001, Everhart and Ruhl, 2009).

2.3.2 TRPV4 antagonist

The TRPV4 antagonist, HC067047, was obtained from TOCRIS (San Diego, CA). Everaerts et al. (2010) have shown that this antagonist is a potent and selective inhibitor of the TRPV4 channel mediated response to heat, arachadonic acid, and 4α-phorbol 12, 13-dihexanoate (4α-PDH). Injections were made intraperitoneally (i.p.) at 10 mg/kg, after which the mouse was returned to the home cage for at least 30 minutes. Testing began at 60 minutes after injection. This time frame was determined as the peak effectiveness using the TRPV4 antagonist dose response curve generated by the authors of the Everaerts paper (Everaerts et al., 2010).

2.4 Histology

Histology was carried out using a modified method described as in our previous paper (Zhang et al., 2014). Mice were perfused transcardially with a 4% paraformaldehyde (PFA) solution after which the entire pancreas was excised and post-fixed in a 4% PFA solution overnight, then changed to 70% alcohol. After dehydration through graded ethanol (95, 100%), the pancreases were embedded in paraffin, and sectioned into 10 μm thickness using a Heidelberg Microm 350 microtome.

2.4.1 Sirius Red Staining for Collagen

Pancreatic sections were deparaffinized and rehydrated. The tissues were stained with Sirius Red (0.1%, EMS Hatfield, PA) and Fast Green was used as a counterstain. Stained tissue sections were examined under light microscopy for changes indicative of pancreatitis, with particular interest in fibrosis. A Nikon Eclipse E1000 microscope was used with a 20X objective to capture random images of five sections from each animal. Computer-aided fibrosis calculation requires the placement of a digital mask over the image, followed by computation of the amount of staining seen. Fibrosis is composed of collagenous material and is stained by Sirius Red. Fibrosis amount was calculated by computer-assisted densitometry using an overlay to determine the density of Sirius Red staining of collagenous proteins present in the tissue.

2.4.2 Hematoxylin and Eosin (H&E) Staining

Slides were de-paraffinized with Citrosolv (Fisher, Pittsburgh, PA), hydrated by down stepping ethanol (100%, 95%, 70%, 50%), rinsed in tap water, and immersed in 0.1% hematoxylin (Fisher, Pittsburgh, PA) for 1 minute. Then slides were washed in tap water for 1 minute, dehydrated by stepping up ethanol percentage (50%, 70%, 95%), immersed in 0.1% eosin (in 95% ethanol, Fisher, Pittsburgh, PA) for 1 minute, and then cleaned and dehydrated in

95% ethanol. Finally, sections were dehydrated in 100% ethanol, then Citrosolv, and cover-slipped with Permount (Fisher, Pittsburgh, PA).

Stained slides were imaged using a Nikon Eclipse E1000 microscope equipped with the ACT-1 program. Five randomly chosen sections from each treatment group were analyzed for tissue morphological changes and fibrosis. All images of the sections used in the analysis were captured with the same background level set based on the control group unstained white area in the section. Illumination levels and microscope settings were maintained through collection of all data samples.

2.5 Statistical Analysis

The data were expressed as means±S.E. Comparisons among groups at different time points or different doses were performed with a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post tests using SigmaPlot version 12.0 (Systat Software, San Jose California, USA). Two-tailed t-tests were also used where appropriate. A $p \leq 0.05$ was considered significant.

3. RESULTS

3.1 AHF Fed Mice Showed Chronic Pancreatitis Morphology with Increased Fibrosis

The pancreatic histology of mice fed the alcohol and high fat diet showed morphological disruption common to CP: atrophy, diffuse nuclear material and poor cellular architecture (Figure 5B) versus the histological uniformity in control mice (Figure 5A and 5C). Fatty infiltration lipid vacuoles were easily seen in the pancreatic tissue of AHF pancreatitis mice along with some tumor-like structures. Increased fibrosis was evident in AHF animals (Figure 6).

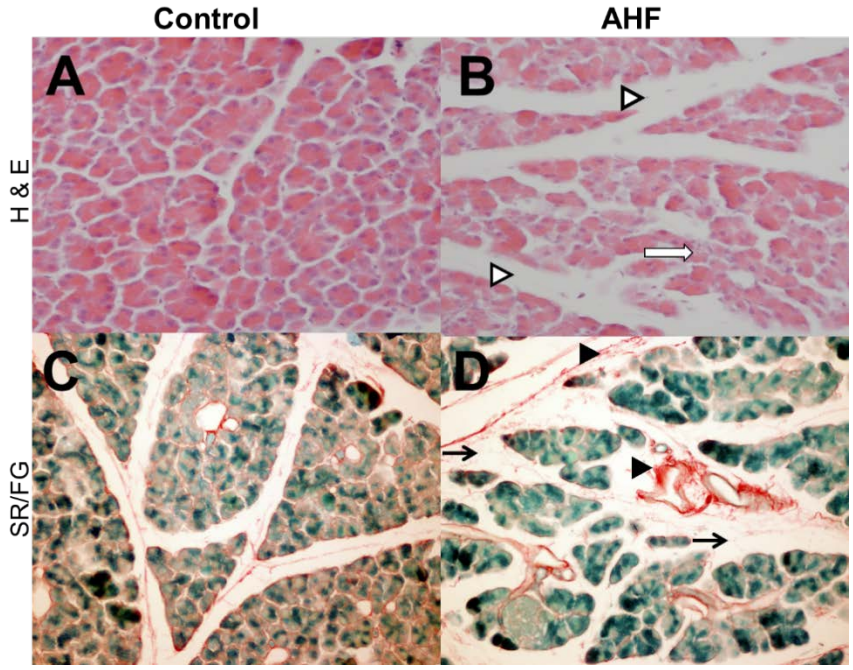

Figure 5. Chronic pancreatitis histopathology is evident in tissue sections from AHF pancreatitis mice. Pancreatic tissue sections from control mice show typical structural morphology (A) and (C). (B) Increased intralobular space (white arrowheads), degradation of acinar cells (white arrow), and (D) fibrosis (black arrowheads) in mice with AHF chronic pancreatitis.

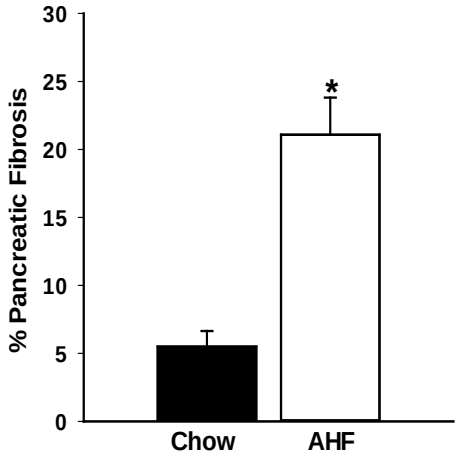

Figure 6. Increased percent fibrosis in AHF pancreatic tissue. Histogram showing significantly increased percent fibrosis of total tissues in AHF pancreatitis mice versus that of control mice ($p<0.02$, two-tailed t-test). Error bars are ±SEM.

Pancreatic histology shows significantly increased fibrosis in sections from AHF pancreatitis mice compared to that of controls. Fibrosis is composed of collagenous material and is stained by Sirius Red. Figure 5 shows representative pancreatic sections from both control (A

& C) and AHF pancreatitis mice (B & D) stained with Sirius Red and Fast Green. Note the atrophy and increased fibrosis in Figure 5C versus 5D. The percent fibrosis of the total tissue was significantly higher in the AHF pancreatitis mice (21±2.7 % of total tissue). In contrast, the percent fibrosis of the total tissue in the normal chow controls was 5.5±1.2 % (Figure 6). There is a significant difference between AHF pancreatitis animals and normal chow controls ($p<0.02$, two-tailed t-test).

3.2 Mice with AHF Pancreatitis Developed Secondary Mechanical Allodynia

Secondary mechanical hypersensitivity was assessed by testing both the paw withdrawal threshold (PWT, Figure 7A) and the rough or smooth mechanical plate (Figures 8 and 9). The 50% hind paw withdrawal thresholds to intermittent mechanical stimulation in the mice were measured over a four week period to establish a baseline prior to drug testing. The 50% paw withdrawal thresholds of mice with CP were significantly decreased (0.49±0.16g) compared to those of normal chow controls (1.33±0.23g; $p<0.05$ by two-way ANOVA, Tukey post hoc test) (Figure 7A).

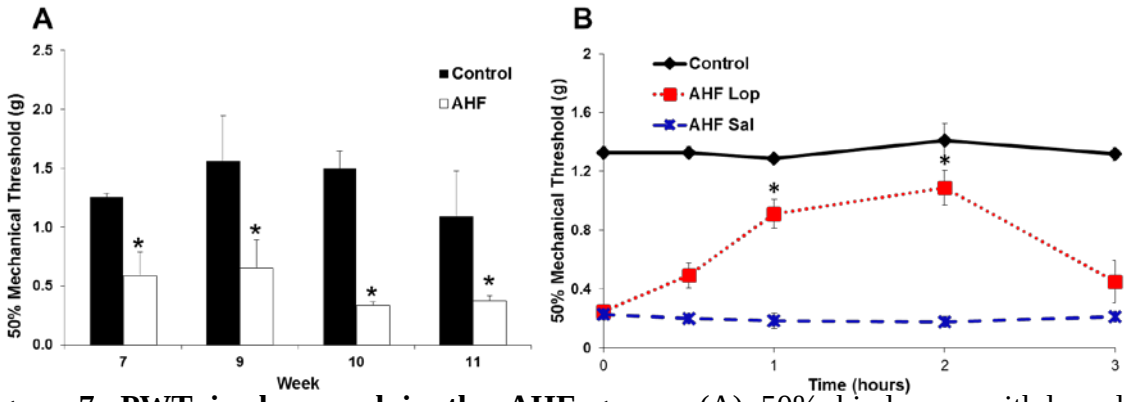

Figure 7. PWT is decreased in the AHF group. (A) 50% hind paw withdrawal thresholds to intermittent mechanical stimulation in the chronic pancreatitis AHF mouse model are significantly decreased versus normal chow controls ($p<0.05$ by two-way ANOVA, Tukey post hoc test, n=6 per group). Loperamide attenuated AHF-induced mechanical hypersensitivity (B). Loperamide treated AHF pancreatitis mice showed a significant elevation in hind paw withdrawal thresholds to intermittent mechanical stimulation compared to saline treated AHF pancreatitis animals at 1hr and 2hr after 0.6 mg/kg dose of Loperamide i.p. ($p<0.05$ by two-way ANOVA, n=4 Control, n=6 AHF). Error bars are ±SE.

3.2.1 Loperamide Attenuated the PWT Secondary Mechanical Hypersensitivity

To test if the hypersensitivity induced by the AHF diet is pain related, the peripherally restricted mu-opioid agonist, Loperamide was given. AHF pancreatitis mice injected i.p. with a 0.6 mg/kg dose of Loperamide HCL showed a significant elevation in hind paw withdrawal thresholds to non-noxious mechanical stimulation compared to that of saline treated AHF pancreatitis animals at 1hr (0.925±0.325g vs. 0.241±0.177g) and 2hr (1.102±0.346g vs. 0.146±0.099g) post injection ($p<0.05$ by two-way ANOVA, n=4 AHF+vehicle, n=6 AHF+LOP). The effect lasted for three hours and peaked at the two hour time point (Figure 7B).

3.2.2 Loperamide Decreases Mechanical Hypersensitivity on the Mechanical Plate

The smooth or rough mechanical plate was used to assess higher brain order responses to the mechanical stimulus. The test device consisted of an acrylic sheet with either a rough diamond pattern providing an uneven surface or a smooth surface that mice were placed upon.

The number of rearing events and rearing duration were recorded using custom software. Mice fed the AHF diet showed a 67% increase in exploratory behavior with intent to escape (rearing events, 39±3.5) on the rough surface sheet (Figure 8A) which gave continuous mechanical stimulation of the plantar hind paws compared to controls (rearing events, 26±3.9; p=0.044 by two-tailed t-test; n=7 Control, n=6 AHF). AHF pancreatitis mice also displayed an increased rearing duration (64±14.5s; n=6) on the rough surface plate (Figure 8B) compared to that of normal chow controls (28±6s; p=0.035 by two tailed t-test; n=7). In contrast, neither the number of rearing events nor the rearing duration showed any significant difference between the two groups on the smooth surface plate.

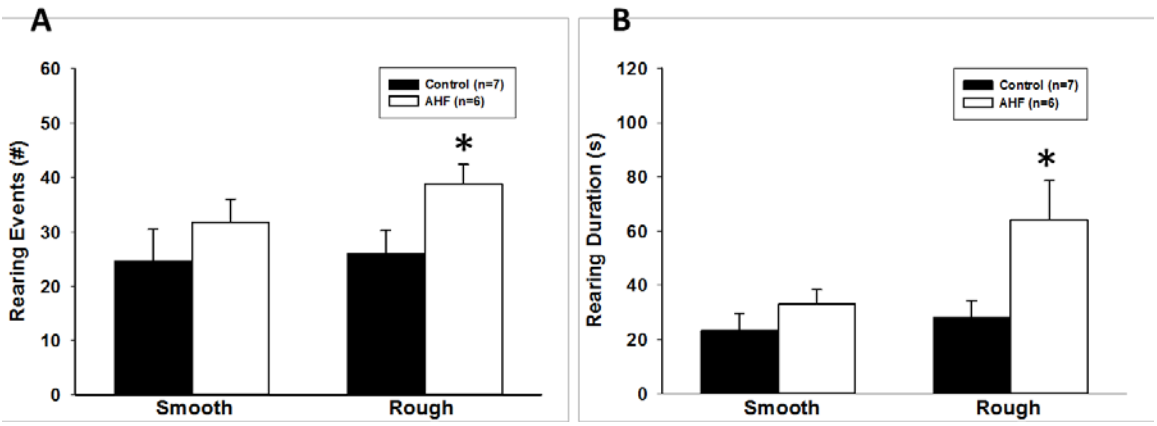

Figure 8. AHF fed mice show elevated baseline escape behavior to rough surface flooring, a mechanical stimulation. Mice fed the AHF diet show a significant increase in number of rearing events (A), and significant prolonged rearing duration (B) to continuous mechanical stimulation of the plantar hind paws compared to that of normal chow controls (p=0.044 and p=0.035 respectively by two-tailed t-test; n=7 Control, n=6 AHF). Error bars are ±SEM. On the non-noxious smooth surface neither the number of rearing events (A) nor rearing duration (B) showed any significant difference between the two groups.

Loperamide HCl i.p. injection at 0.6 mg/kg decreased treated AHF group rearing events and duration significantly relative to the uninjected AHF control group (Figure 9). This indicates, as seen in the intermittent mechanical stimulation of the von Frey test, that escape behavior of

AHF mice to continuous mechanical stimulation experienced on the rough mechanical plate is pain-related.

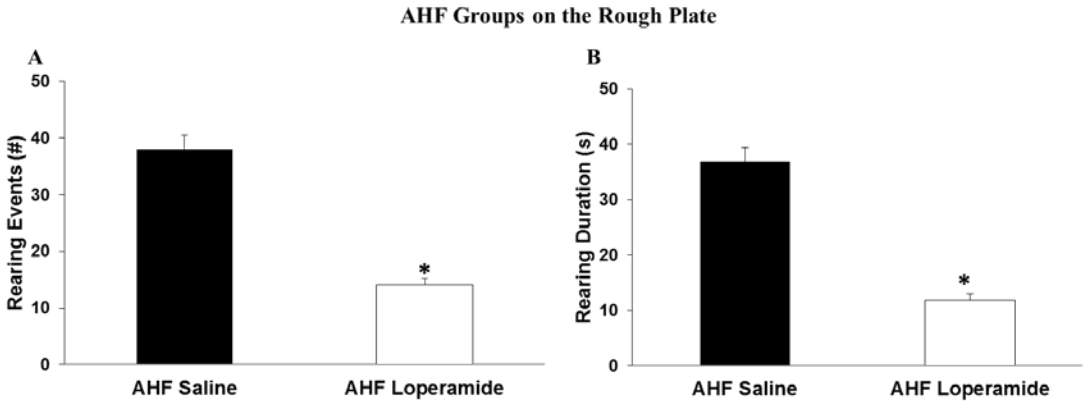

Figure 9. Loperamide HCL treatment decreases rearing and rearing duration in AHF Mice. AHF mice treated with Loperamide display significant reversal of both increased rearing events (A), and rearing duration (B). (p=0.045 and p=0.051 respectively by two-tailed t-test; AHF Control n=6, AHF Loperamide n=6).

3.3 AHF Pancreatitis Mice Developed Nocifensive Behaviors on Hot Plate Testing

The modified 44°C hotplate was used to determine heat sensitivity of mice in the study. The number of rearing events at baseline testing of 38°C showed little difference between AHF pancreatitis and normal chow control mice (23±6.8 vs. 20±6.7 rearing events) (Figure 10A). AHF pancreatitis mice, however, showed a significantly increased (55%) number of rearing events (60±8 events) (indicating escape behaviors) on the modified 44°C hotplate than did normal chow control mice (33±2; p=0.011; by two tailed t-test) (Figure 10B).

3.4 Loperamide Attenuated the Nocifensive Response to the 44°C Hotplate Stimuli in a Dose Dependent Manner

Baseline rearing events at 38°C showed little difference between AHF pancreatitis and normal chow control mice (Figure 10A). AHF pancreatitis mice showed a significantly greater number of rearing events (60±7.5) on the baseline modified 44°C hotplate than did normal chow Control mice (33±1.5) (Figure 10B). Systemic Loperamide (i.p.) produced a dose-dependent

decrease in AHF pancreatitis mouse rearing events (0.4 mg/kg; 37±5, 0.8mg/kg; 31±4 events, 1.2mg/kg; 28±5). Vehicle treated AHF pancreatitis mice showed a significantly higher number of rearing events at 44°C than did the Loperamide 1.2mg/kg dose treatment group (38±4), ($p<0.05$ by two-way repeated measures ANOVA (Figure 10C).

3.5 HC067047 Significantly Shifted the Event-time Curve Right-ward

The TRPV4 antagonist, HC067047 (10mg/kg; i.p.), significantly decreased the number of jump events (52%) in AHF pancreatitis mice in modified 44°C hotplate testing 13±5.3, (AHF+HC, n=6) vs. 25±10.2 (AHF+vehicle, n=7); ($p=0.028$ by two-tailed t-test). The latency to first jump event was prolonged in HC067047 treated AHF pancreatitis mice by 51% (321±111s (AHF+HC) vs. 136±58s (AHF+vehicle)). The number of jump events decreased while the latency to first jump was prolonged in AHF pancreatitis animals treated with HC067047. The event-time curve showed a significant right-ward shift (Figure 10D).

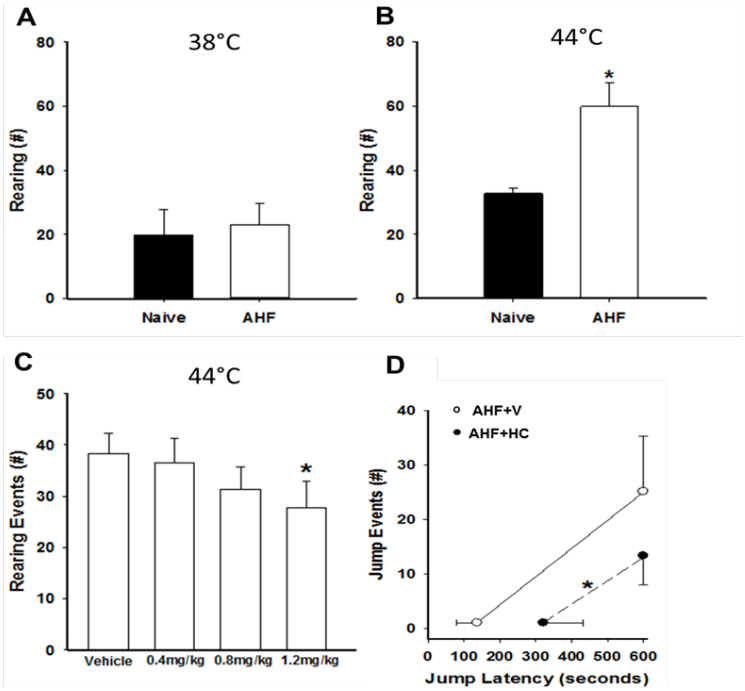

Figure 10. AHF pancreatitis mice exhibit an increase in rearing events in response to heat stimulation at 44°C. (A) The number of baseline rearing events at 38°C showed little difference between AHF pancreatitis and normal chow control mice. (B) AHF pancreatitis mice showed significantly more rearing events on the continuously stimulating modified 44°C hotplate at baseline than did normal chow control mice ($p=0.011$; by two tailed t-test). (C) AHF pancreatitis mice with systemic Loperamide treatment showed decreases in the number of rearing events in a dose-dependent manner (at 1.2mg/kg dose, vs. AHF+Vehicle, $p<0.05$ by two-way repeated measures ANOVA). (D) The TRPV4 antagonist, HC067047 significantly prolonged the latency to first jump in AHF pancreatitis mice versus vehicle treated AHF pancreatitis mice on the 44°C modified hotplate ($p<0.05$ by two-way repeated measures ANOVA, Tukey's Test; n=7 AHF+Vehicle; n=6 AHF+HC). The event-time curve showed a significant right-ward shift. Error bars are ±SE.

4. DISCUSSION

The aim was to produce an alcohol and high fat induced mouse model of CP for laboratory study. Models of CP have been called for by those prominent in the field of pancreatitis research (Witt et al., 2007). The diet induced model discussed here was intended to be effective in nontransgenic mice to produce a model of CP that more closely resembles the development of the disease in humans. The epidemiology of pancreatitis shows two times the

chance of diagnosis of the disease in overweight and obese people; it also shows that of those with CP, 70% abuse alcohol (Martinez et al., 2004, Siech et al., 2009). This model, incorporating alcohol and high fat, provides two long term “hits” to the system of the mouse and produces a deleterious burden in body homeostasis leading to tissue damage (CP) with pain related complications.

In the characterization of a new model, showing that the model has the disease targeted effects is an important first step. The model was reliably produced in all mice fed the AHF diet. Histology conducted on the pancreas of AHF pancreatitis mice showed morphology common to CP: fibrosis, diffuse nuclear material, poor cellular architecture, and fat vacuole formation. Increased fibrosis is a common feature in CP due to the activity of pancreatic stellate cells (PSC). In the presence of alcohol and fatty acids, these normally quiescent PSCs become activated (Zhang et al., 2013). Activated PSCs synthesize and secrete endogenous cytokines which influence PSC function via autocrine pathways. This leads to excessive extracellular matrix (ECM) production, eventually causing wide spread pancreatic fibrosis, and the persistent damage seen in CP. Percent pancreatic fibrosis in the AHF pancreatitis mice was significantly greater than that of normal chow controls. The increased fibrosis in AHF pancreatitis mice likely indicates that PSCs were activated and overproducing ECM within the pancreas of AHF pancreatitis mice.

Correspondent to the morphological changes within the pancreas, AHF pancreatitis mice showed hypersensitive behaviors in both mechanical and thermal testing. AHF pancreatitis mice displayed secondary mechanical allodynia on their hind paws as demonstrated by the decreased paw withdrawal threshold. Correspondingly, AHF pancreatitis mice showed significantly increased numbers of rearing events on the rough surface at baseline. AHF pancreatitis mice also

exhibited significantly increased numbers of rearing events on the 44°C modified hotplate. Vierck et al., (2002) determined that temperatures from 43°C to 45°C can provide C fiber activation. Chronic pain in the clinic has been associated with prolonged activation of the C-nociceptors (Yeomans et al., 1996).

Laboratory animal drug testing has traditionally been concerned with relieving acute pain stimuli such as that seen on the hotplate test. Acute testing tends to focus on pain carried by the Aδ afferents, also known as "first pain". Chronic pain is a significant problem for patients in the clinic and one that is most important to address. Slow increases of nociceptive stimuli such as heat tend to replicate the type of pain seen in humans in the clinic as it activates C-fiber nociceptors preferentially.

The reality of first and second pain show that all pain is not alike. First pain is immediate upon exposure to a painful stimulus. Descriptors of this pain in humans include "sharp" or "stabbing". First pain is carried by myelinated Aδ fibers. The myelination allows quick movement of the afferent pain signal to the brain. Aδ fibers conduct signals at a speed of (5-30 m/s) to the brain. Second pain is delayed pain felt after first pain subsides. Descriptors of this pain in humans include "dull" or "throbbing". Second pain is carried by small unmyelinated C-fibers. The lack of myelination means slow movement of the afferent pain signal to the brain. Indeed, the C-fiber carries signals at a rate of (0.5-2 m/s) to the brain, compared to the A delta fibers at (5-30 m/s).

Drugs that have been tested against first "pain" in laboratory animal trials don't always prove effective in treating second "pain". This is because first pain activates the A delta nociceptors while second pain activates the C-fibers. Second pain in laboratory animals is comparable to chronic clinical pain in humans. Locating analgesics specific to the nociceptive

fiber responsible for “pain” signaling is expected to provide relief to humans and a decrease in pain-like behaviors in laboratory animals. The nociceptors actively targeted in the modified hotplate procedure are specific to C-fibers. Vierck et al., (2002) determined that temperatures from 43°C to 45°C can provide C fiber activation.

C-fibers were preferentially activated during testing of the analgesic properties of HC 067047, or Loperamide HCl. HC067047 showed a trend of effectiveness against heat induced nociceptive stimuli. HC067047 could therefore be active against pain in humans as it treats C-fibers in mice.

The 44°C hot plate stimulus is considered a noxious continuous stimulation. The AHF pancreatitis mice had an increased nocifensive response to the 44°C modified hotplate stimulus, i.e. jumping. The increase in jumping responses are indicative of secondary heat hyperalgesia. This pancreatitis associated secondary hypersensitivity can be effectively attenuated by systemic application of either Loperamide HCL, a peripherally restricted mu-opioid agonist; or HC06704, a TRPV4 channel antagonist.

Loperamide was used to avoid central effects as we only wanted to affect the peripheral nerve innervation of the pancreas (Khawaja et al., 1990, Khalefa et al., 2012). The use of pain relief drugs in humans can cause impairments as the drugs cross the blood-brain barrier (Ringkamp and Raja, 2012). The peripherally restricted action of Loperamide is meant to avoid effects of central impairment in mice in the study, and later in humans. Therefore, Loperamide was chosen to agonize the mu-opioid receptors in the peripherally-located damaged pancreatic tissue.

Loperamide is best known for its antidiarrheal properties, but it has found limited use as a peripherally restricted agonist of the mu-opioid receptor (MOR) system (Kumar, 2013).

Loperamide is a non-addicting, mu-opioid agonist expected to bind mu-opioid receptors in the pancreas, inhibit neurotransmitter release, and decrease calcium influx to the cell. Mu-opioid receptors are increased in damaged pancreatic tissue (Lu et al., 2007). Loperamide likely provided analgesic effects by decreasing nociceptive signaling through mu-opioid receptors on pancreatic nerves. Blunting the nociceptive stimulation from the pancreas by MOR activation was expected to decrease mouse mechanical and thermal hypersensitivity.

In this study, antagonism of TRPV4 channels in AHF pancreatitis animals attenuates nocifensive behaviors. The first jump latency is significantly prolonged, while the number of jump events on the hotplate decreases in HC067047 treated AHF pancreatitis mice. This is indicative of decreasing escape behavior following TRPV4 antagonist treatment. Everaerts (2010) determined that HC067047 produced potent and selective inhibition of TRPV4 channel activity in a bladder inflammation model. Zhang et.al (2013) have shown that the expression of the TRPV4 channel is increased in activated pancreatic stellate cells in the AHF CP rat model. Miranda et al. (2014) have shown the presence of TRPV4 in the pancreatic dorsal root ganglion as well as pancreatic nerve fibers. In this study, the enriched metabolites of alcohol and fatty acids likely activate TRPV4 receptors in the nerve endings, making them spontaneously active, or more readily activated, causing an amplification of nociceptive signaling. Following antagonism of TRPV4 channels, the prolonged first jump latency and decrease in number of jump events is denoted by an event-time curve right-ward shift similar to that seen following treatment with morphine (Dirig and Yaksh, 1995).

5. CONCLUSION

The data presented here are evidence supporting the successful production of an AHF diet alone induced mouse model of CP. The successful reduction of pain related behaviors by

both Loperamide and HC067047 suggest that the CP model showed responsiveness to activation of opioid receptors, as well as increased activation of TRPV4 channels in the pancreas and peripheral nervous system. Loperamide, a peripherally restricted mu-opioid receptor agonist, attenuated both mechanical and heat hypersensitivity in the mouse CP model. The TRPV4 antagonist, HC067047 reduced heat hypersensitivity. This implies that either TRPV4 antagonism, or agonizing the peripheral opioid system (Loperamide) can be used to inhibit visceral pain signaling associated with CP.

6. ACKNOWLEDGEMENTS

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CHAPTER THREE

Anxiety- and Fear-like Behaviors in a Mouse Alcohol and High Fat Chronic Pancreatitis Model

ABSTRACT

Background/Aims: The previous study characterized pain related responses in an Alcohol and High Fat (AHF) diet-induced mouse model of CP. The aim of this study is to characterize anxiety-like behaviors in this chronic pain mouse model.

Methods: Mice were fed normal chow ad libitum or a liquid diet containing 6% alcohol and a high fat supplement over a period of five months. Anxiety-like behavior, escape behavior, and locomotion were measured using the following: Place Preference testing including the light/dark box and 44°C/22°C escape test; plus maze, open field, the smooth or rough mechanical plate, and a modified 44°C hotplate procedure. Pharmacological agents tested in the modified hotplate test were a peripherally restricted mu-opioid (Loperamide HCl) and a TRPV4 antagonist.

Results: Overall AHF mice have increased anxiety-like behaviors displaying both more exploration/escape responses and increased locomotion. The AHF mice also show increased fear-like freezing behavior on the modified 44°C hotplate. In the open field test AHF mice showed similar mobility and decreased escape behaviors relative to controls.

Conclusion: A test battery including light/dark place preference, and 44°C/22°C escape testing, plus maze, open field, and the modified hotplate can be used to better understand anxiety-like behavior in a mouse model of CP. AHF mice display increased anxiety-like behaviors, providing an experimentally analogous model of the anxiety shown by patients diagnosed with CP pain.

1. INTRODUCTION

The correlation of CP hypersensitivity with anxiety and stress-induced analgesia (SIA) has been understudied. Nineteen million adults (18%) in North America experience anxiety disorders (Cryan and Holmes, 2005) while 35% of patients in chronic pain have anxiety (McWilliams et al., 2003). Up to 75% of people diagnosed with CP experience terrible pain as the disease progresses (Di Sebastiano et al., 2000, Madan et al., 2013). Therefore, the percent anxiety in the population of patients with CP is likely to be higher than that seen in the general public. While data concerning the percent of patients with CP pain comorbid with anxiety are not available, decreased QoL is well established in pancreatitis patients (Pezzilli et al., 2005, Mullady et al., 2011, Walsh et al., 2012, Amann et al., 2013). Patients with anxiety experience lower QoL (Brenes, 2007). People with CP experience anxiety (Walsh et al., 2012). Using a battery of tests to determine the presence of anxiety-like behaviors in a mouse model of CP allows exploration of CP mouse model characteristics that mirror those of patients diagnosed with CP. Increased similarity between the patient and mouse model increases translatability to clinical patients with CP.

Anxiety, like pain, is useful as a transient state but when anxiety is prolonged it is termed a disorder. Anxiety of this kind is characterized by “a marked, persistent, & excessive or unreasonable fear” (DSM-4) that interferes with normal life (Roy-Byrne et al., 2008). Steimer (2002) suggested that anxiety is defined as “a psychological, physiological, and behavioral state induced in animals and humans by a threat to well-being or survival, either actual or potential.” Anxiety is often comorbid with pain and depression (Kroenke et al., 2013). Studies of life stress have shown that it accelerates the progression and severity of diseases such as cardiovascular disease (CVD) (Krantz and McCeney, 2002), HIV (Leserman et al., 2002), and depression

(Hammen, 2005). Both chronic stress and anxiety can lead to and be comorbid with decreased health (Roy-Byrne et al., 2008, Tomiyama et al., 2011). It is critical to determine the part anxiety and stress play in the observed pathology of CP because CP patients display increased anxiety (Walsh et al., 2012).

Stress-induced analgesia (SIA) occurs when exposure to stressful stimuli causes suppression of pain. SIA occurs in humans as well as other species such as the mouse. SIA is activated upon intense stimulation of pain-related circuitry (Rhudy and Meagher, 2000), such as after receiving an injury in war (Beecher, 1946), or during a marathon (Janal et al., 1984). SIA has also been observed in animals. Anxiety-like behaviors in rodents are easier to detect after 6-8 weeks of chronic pain (Yalcin et al., 2011, Yalcin et al., 2014). SIA in the rodent can be induced in a number of ways including exposure to electrical foot shock, or hotplate (Amit and Galina, 1986, Butler and Finn, 2009). It is thought to be an evolutionary survival response allowing an injured animal to escape further injury by deadening a distracting immediate response to algesic stimuli such as bodily injury (Butler and Finn, 2009). SIA gives the animal time to leave the dangerous environment where it received injury. To test the induction and presence of SIA in the AHF CP mouse model, we will use both the 44°C hotplate, and the 44°C/22°C Escape Place Preference test.

A variety of measures were used to test anxiety in the AHF CP mouse model including testing in the Open Field, Plus Maze, Smooth or Rough Mechanical Plate, 44°C Modified Hotplate, Place preference Light/Dark Box, as well as a 44°C/22°C Escape test. These tests are designed to assess anxiety-like behaviors, stress-induced analgesia, mechanical and thermal hypersensitivity, and spontaneous exploration behavior. We anticipated that AHF-diet induced

CP mice would display increased anxiety-like behavior and stress-induced analgesia relative to control mice.

2. METHODS

The studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.1 Induction of Pancreatitis

Five month old male mice on a C57BL/6 background weighing less than 40g (The Jackson Laboratory) were used for this study. Animals were housed in a temperature controlled room maintained at between 21-24°C, on a reverse light:dark 14:10 hour schedule. Control mice were fed rodent chow (Mouse Breeder Diet 8626) from Teklad (Madison, WI). AHF pancreatitis mice were fed a Lieber-DeCarli liquid diet from Test Diet (LD101A, Richmond, IN) and supplementary fat. The total fat content was about 57%. Alcohol was gradually added to the diet over a period of weeks from zero to 4%, 5%, and then 6%. AHF fed mice were maintained at 6% alcohol for the remainder of the study. Body weight was monitored weekly.

2.2 Place Preference and Escape Testing

Place preference and escape testing were conducted in a plastic box 22.8 cm x 15.2 cm x 16.5 cm (9 in L x 6 in W x 6.5 in H) Great Choice Critter Tote (PetSmart) with dime sized air holes drilled in the top. A darkened divider with a door measuring 5.08 x 5.08 cm (2 x 2 in) was added to the box, dividing it into two equal chambers each measuring 11.4 x 7.6 x 8.3 cm (4.25 x 6 x 6.5 in). Each place preference or escape test had a duration of 10 minutes.

2.2.1 Light/Dark Place Preference

Test apparatus as in 2.2 was used for this test with one chamber of the box darkened, while the other chamber was well lit. In addition, free movement between the light and dark chambers was permitted through the 5.08 x 5.08 cm (2 x 2 in) opening.

2.2.2 44°C/22°C Escape

Test apparatus as in 2.2 was used for this test. One chamber of the test apparatus had a floor regulated to 44°C and was darkened. The other chamber had room temperature flooring at 22°C±1°C with bright light. In addition, free movement between the light and dark chambers was permitted through the 5.08 x 5.08 cm (2 x 2 in) opening. Mice were added to the 44°C side of the box and grooming, transitions, rearing, and latency to rear were recorded.

2.3 Smooth or Rough Mechanical Plate

The test apparatus consisted of a clear Plexiglas box (11cm X 8cm X 8cm) placed upon either a smooth or roughly textured surface. Rearing exploratory activity, including frequency and duration, with or without a rough textured floor insert (the textured side of a polystyrene ceiling light panel diffuser (89091, Lowes.com), was monitored in real time and captured using custom software during 5 min tests.

2.4 Plus Maze

Testing on the plus maze was modified from that previously described and will be described here (Lyons et al., 2015). The elevated plus maze (Bioseb, Vitrolles, France) consists of four arms arranged in a cross-shaped design (length: 35 cm, width: 5 cm/each, height from floor: 51 cm). Two arms are enclosed on three sides by 15-cm high walls and the other two are not. All arms meet in a central area (5 cm × 5 cm) which allows animals to move freely throughout each zone of the maze. Mice were initially placed in the central area of the maze and

allowed to explore the maze for a period of 5 min. Mouse behaviors were recorded and analyzed for: (1) time spent in open arms, (2) number of transitions into the open and closed arms, and (3) number of head dips into the open arms, defined as the movement of the animals head from the closed arm to the open arm of the maze. In this test, the open arms represent a potentially threatening environment.

2.5 Open Field

Testing in the Open Field was completed as previously described in Lyons et al. (2015), and will be described here. Exploratory behaviors were measured using a Flexfield Animal Activity System (San Diego Instruments, San Diego, CA, USA). This apparatus consists of two Plexiglas chambers (40 × 40 × 36 cm) equipped with Photobeam Activity System (PAS) software coupled to a Compaq 486 computer (Hewlett Packard, Palo Alto, CA, USA). Each chamber contained infrared photobeam sensors with 16 beams on each axis (total of 32 beams) that are arranged 1.25 cm above the chamber floor. Obstruction of these photo beams constituted movements in the *x* - and *y* -planes. The *x* - and *y* -planes were further divided into a central and peripheral area. Another set of 16 beams is located 8 cm above the chamber floor to record movements along the *z* -axis measured i.e. rearing events and rearing duration (Zhang et al., 2004). Data were collected in 5-min intervals for a total of 45 min to record: (1) number and duration of rearing events (2) active time vs. rest time, (3) overall distance traveled, (4) total beam breaks, and (5) time spent in the central verses peripheral areas of the chamber.

2.6 Modified 44°C Hotplate Assay

This procedure was performed as in Kline and Wiley (2008), and will be described here. In modified 44°C hotplate testing, mice are first placed on a 38°C hotplate for ten minutes in order to slowly pre-warm the animal's feet. Thereafter the mice were moved to a 44°C hotplate

for a ten-minute testing period (Kline and Wiley, 2008). In this study, jumping events, rearing events, and latency to first jump were the dependent variables and were recorded using custom software.

2.7 Drugs

2.7.1 Mu-opioid Receptor Agonist

Loperamide HCl (SIGMA-Aldrich, Milwaukee, WI) is a peripherally restricted, mu-opioid receptor (MOR) agonist commonly used to treat diarrhea (Awouters et al., 1993). Loperamide HCl was suspended in a 20% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Milwaukee, WI) in saline and diluted to the proper dose concentration. Loperamide HCl injections were made intraperitoneally (i.p.) at 0.4, 0.6, 0.8, or 1.2 mg/kg and testing began at 60 minutes after injection (Reichert et al., 2001, Everhart and Ruhl, 2009).

2.7.2 TRPV4 antagonist

HC067047 is a potent and selective TRPV4 antagonist (Tocris, San Diego, CA). Everaerts et al. (2010) have shown that this antagonist inhibits TRPV4 channel mediated responses to heat, arachadonic acid, and 4α-phorbol 12, 13-dihexanoate (4α-PDH). Injections were made intraperitoneally (i.p.) at 10 mg/kg, after which the mouse was returned to the home cage for at least 30 minutes. Testing began at 60 minutes after injection. This time frame was determined to be within the peak effective time period using the TRPV4 antagonist dose response curve generated by the authors of the Everaerts paper (Everaerts et al., 2010).

2.8 Statistical Analysis

The data were expressed as means±S.E. Comparisons among groups at different time points or different doses were performed with a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post tests using SigmaPlot version 12.0 (Systat

Software, San Jose California, USA). Two-tailed t-tests were also used where appropriate. A $p \leq 0.05$ was considered significant.

3. RESULTS

3.1 Persistent Visceral Hypersensitivity Causes Stress Induced Analgesia.

Testing of the 44°C/22°C escape response shows progressively increasing anxiety-like behavior from the eighth to tenth weeks as visceral hypersensitivity AHF CP model mice display significantly increased average transitions (see **Figure 11**) (week 8, control=46, AHF=64; week 9, control=44, AHF=89; week 10, control=26, AHF=66; p=0.029, 0.031, 0.036 by two-way ANOVA and Tukey's; n=6, n=6). Cumulative transitions become significant in the last three weeks of testing (Weeks 8-10) (p=0.029, 0.031, 0.036; by 2-way ANOVA and Tukey Test). Increased transitions between test chambers suggest stress has increased relative to untreated controls undergoing the same testing.

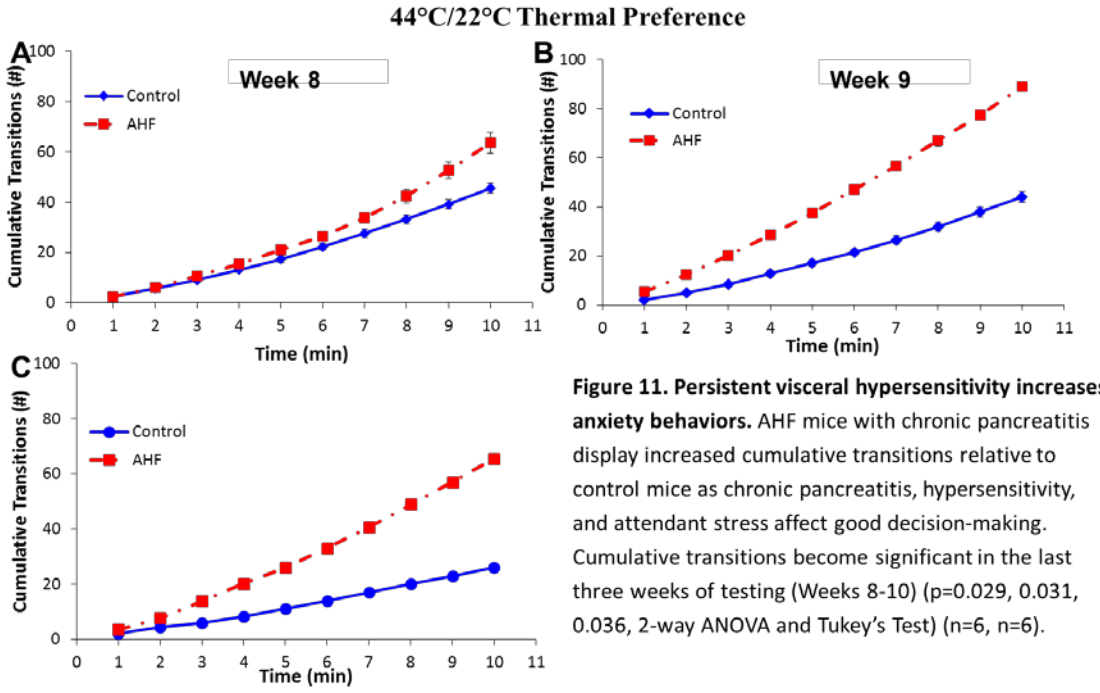

Figure 11. Persistent visceral hypersensitivity increases anxiety behaviors. AHF mice with chronic pancreatitis display increased cumulative transitions relative to control mice as chronic pancreatitis, hypersensitivity, and attendant stress affect good decision-making. Cumulative transitions become significant in the last three weeks of testing (Weeks 8-10) (p=0.029, 0.031, 0.036, 2-way ANOVA and Tukey's Test) (n=6, n=6).

AHF mice displayed behavior indicative of chronic visceral hypersensitivity. Hypersensitivity is a stressor which could be causing the mice to increase exploratory behavior, with an intent to escape. In the seventh week average latency to 1st transition is increased significantly in the AHF animals indicating decreased locomotion in the AHF animals versus controls (see **Figure 12)** (control=1, AHF=3, p=0.031 by two-tailed t-test; n=6, n=6). This decreased initial activity is lost as transitions begin to increase significantly in the eighth week.

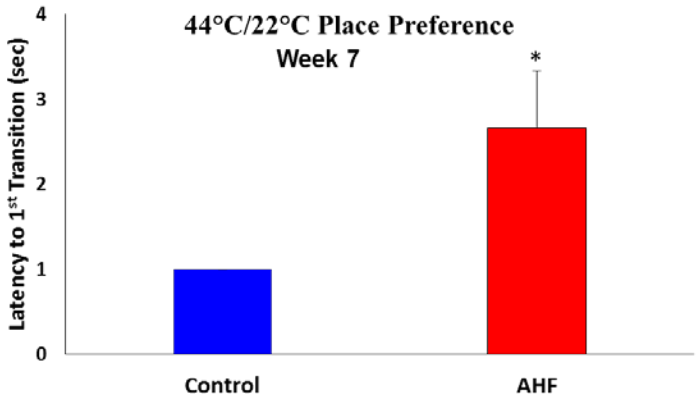

Figure 12. Latency to initial activity is increased in AHF mice. AHF mice have significantly decreased initial activity in the seventh week. Initial activity is not significant by Students t-test in the eighth through the final week of place preference testing. (t-test p=0.031) (n=6)

3.2 Visceral hypersensitivity increases transitions in the Light/Dark Place Preference test (LD).

In the LD, increased transitions between light and dark chambers indicate an increase in exploratory behavior (Bourin and Hascoet, 2003). AHF mice display increased exploratory behavior in week nine. In the last week of testing AHF mice show a significant increase in transition events relative to control mice (Figure 13) (by two-way ANOVA and Tukey's Test).

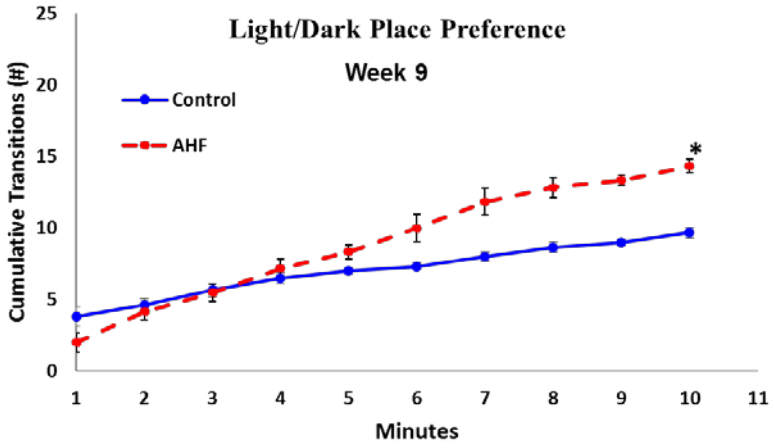

Figure 13. Visceral hypersensitivity increases transitions in the Light/Dark Place Preference test. In week 9 of testing AHF mice show a significant increase in transition events relative to control mice (p=0.026 by two-way ANOVA and Tukey's test). (n=6)

3.3 Grooming behavior changes on the rough surface.

Grooming is a displacement behavior, increased grooming typically indicates increased discomfort due to anxiety (van Gaalen and Steckler, 2000, Steimer, 2011). AHF mice show little change in grooming events from smooth to rough surface, regardless of treatment with either Loperamide or vehicle (**Figure 14A).** Normal chow control mice that are not experiencing visceral hypersensitivity display fewer grooming events from smooth to the rough surface. This decrease in grooming events is not significant, but is of note as grooming does not change in the AHF mice. In a similar manner, grooming latency increases significantly in saline or Loperamide-treated normal chow controls from smooth to rough surface testing (**Figure 14B**). There is no significant grooming latency increase in AHF mice from smooth to rough mechanical plate. AHF mouse Freeze Latency on the smooth surface increases significantly upon Loperamide treatment (**Figure 14C**). Freeze latency is not significantly different on the rough surface in Loperamide-treated AHF mice **(Figure 14D)**. Control mouse latency to freeze remains

constant at 193±7 seconds while AHF mice decrease 49 seconds on the rough surface compared to the smooth surface **(compare Figure 14C and 14D)**.

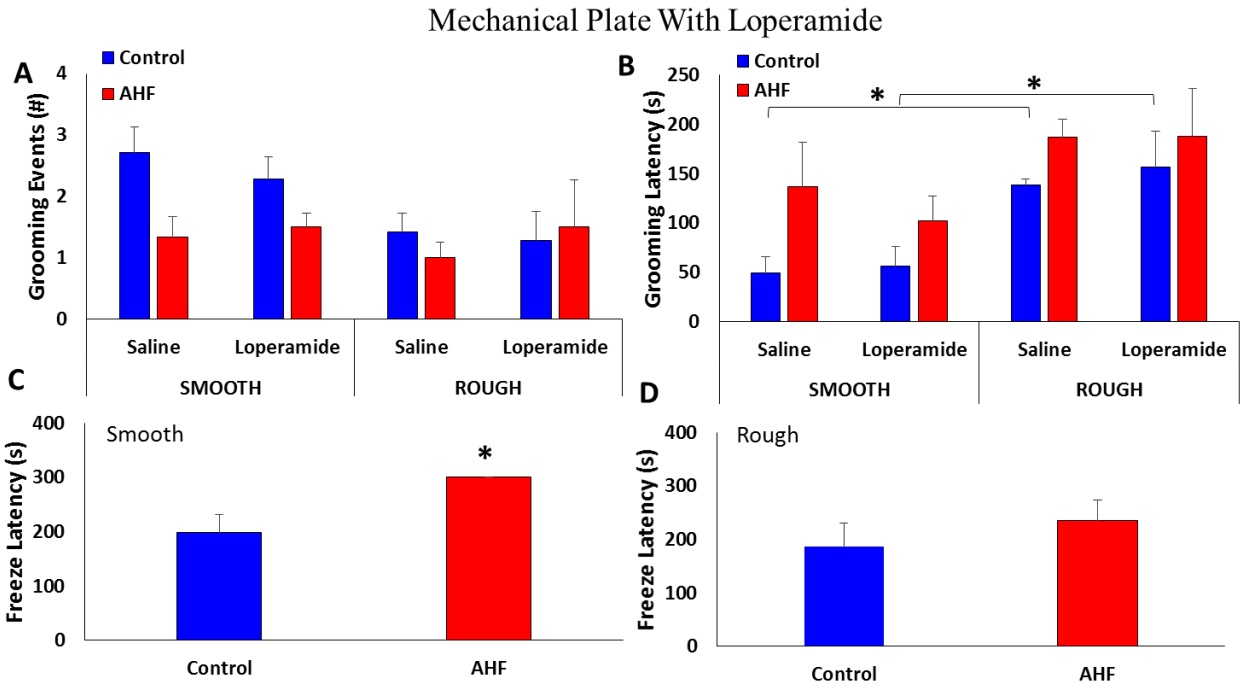

Figure 14. Grooming behavior changes on the rough surface. (A) AHF mice show little change in grooming events from smooth to rough surface. (B) Grooming latency increases significantly only in normal chow controls during the transition from smooth to rough surface testing. AHF mice show an insignificant increase in grooming latency from the smooth to rough plate. (C) Loperamide treatment significantly increases AHF mouse freeze latency on the smooth surface. (D) Freeze latency on the rough mechanical plate decreases in AHF mice with little change seen in control mice. (n=6, AHF all groups; n=7, All normal chow Control)

3.4 AHF mice increased exploration in the Plus Maze.

The number of closed arm entries is a measure of locomotion in the plus maze (Campos et al., 2013). An increase in number of closed arm entries on the elevated plus maze shows significantly increased exploration and locomotor activity in the AHF mice relative to controls **(Figure 15A)** (p=0.052, two-tailed t-test). The percentage of open arm head entries is considered a measure of anxiety-like behavior in the plus maze (Campos et al., 2013). AHF mice tested the

open arms with head entries significantly more than controls **(Figure 15B)** (p=0.043, two-tailed t-test).

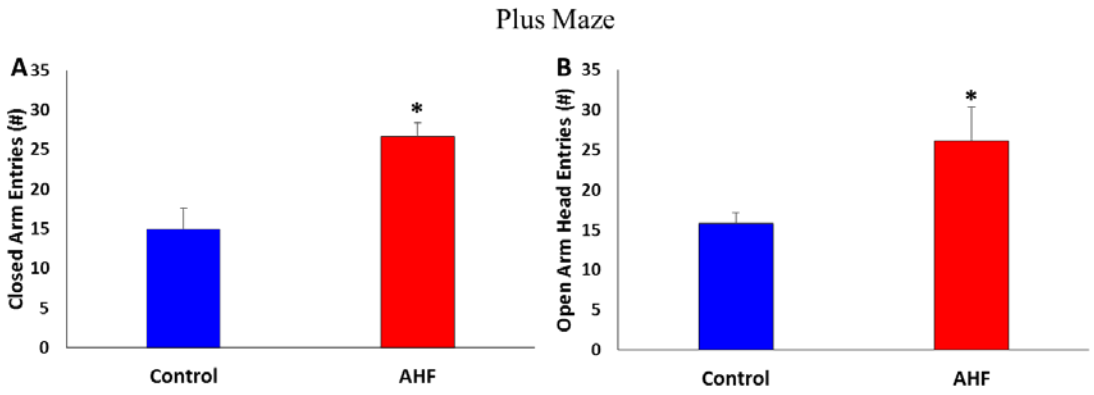

Figure 15. AHF mice increase exploration in the plus maze. (A) Significantly increased cumulative closed arm entries on the elevated plus maze indicate increased exploration and locomotor activity in the AHF mice. (B) Significantly increased open arm head entries indicate higher anxiety in the AHF mice.

3.5 AHF mice display decreased escape behavior in the Open Field test.

AHF mice display significantly decreased rearing events **(Figure 16A and B)** (p=0.0045 by two-tailed t-test) and rearing duration (p=0.0045 by two-tailed t-test) in the open field test compared to vertical activity of the controls. Mice with anxiety-like behaviors display thigmotaxis, a state where the animal stays close to the walls of the test apparatus, avoiding the center (Simon et al., 1994).

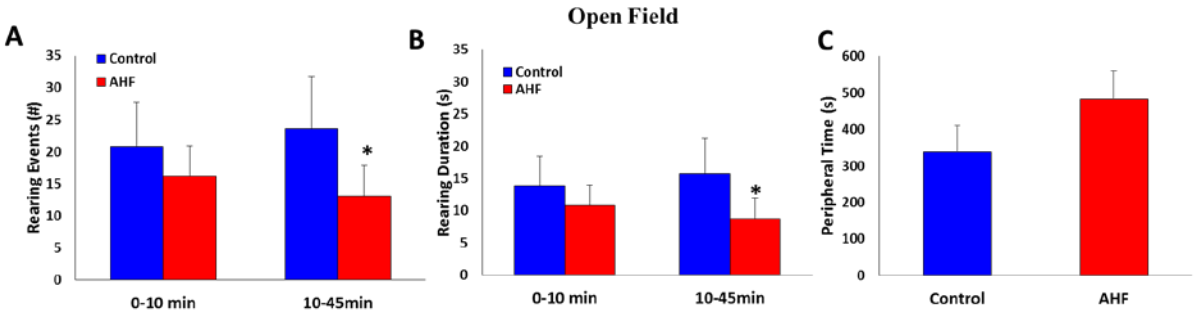

Figure 16. AHF mice display decreased escape behavior in the Open Field. (A) AHF mice display significantly decreased rearing events in the open field test. (p=0.0045 by two-tailed t-test) (B) AHF rearing duration was significantly decreased in the open field test (p=0.0045 by two-tailed t-test). (C) AHF mice show a trend toward increased thigmotaxis in relation to control mice.

3.6 AHF mice increased freeze events on the 44°C modified hotplate.

A mouse freezes in response to a fear-provoking stimulus (McIlwain et al., 2001, Campos et al., 2013). Freezing is defined as the absence of any movement, except for respiration, generally for a period of 5-10 seconds (Paylor et al., 1994). In **Figure 17**, the modified hotplate test provides a contextual stimuli. Vehicle treated control mice display more freeze events than in AHF mice. AHF mouse freeze events show no significant freezing change between Loperamide doses at 0, 0.4, and 0.8 mg/kg. AHF mouse freeze events are significantly increased at a Loperamide dose of 1.2 mg/kg (p=0.025 by two-tailed t-test, n=7, n=6).

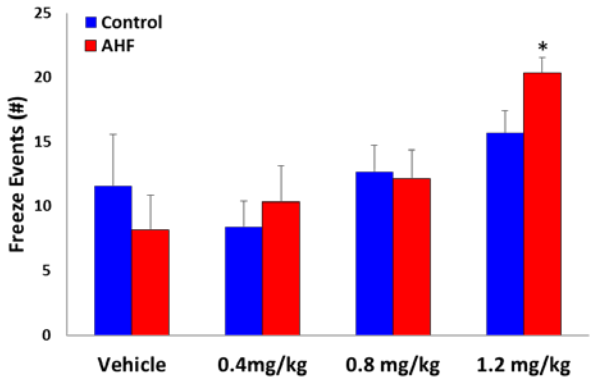

Figure 17. AHF mice display increased freeze events on the modified hotplate. AHF mice freeze events increase significantly at a loperamide dose of 1.2 mg/kg (p=0.0022 by two-tailed t-test). Control mice show little difference in freezing.

3.7 Low bodyweight increases thigmotaxis in AHF mice.

By study week five the AHF treatment group split into a high and a low bodyweight group **Figure 18A**. AHF mice 1, 2, and 4 regularly consumed their lard supplement and at study endpoint weighed 45 g on average. AHF mice 3, 5, and 6 consumed less lard and at study endpoint weighed 34 g on average **Figure 18B**. All AHF average combined mouse bodyweight was 40 g. Chow fed control mice weighed on average 38 g at study endpoint. Mice with anxiety-like behaviors display thigmotaxis, a state where the animal stays close to the walls of the open field test apparatus, avoiding the center (Simon et al., 1994). AHF mice with lower bodyweight explored the open field periphery more than did higher bodyweight mice **Figure 18C**.

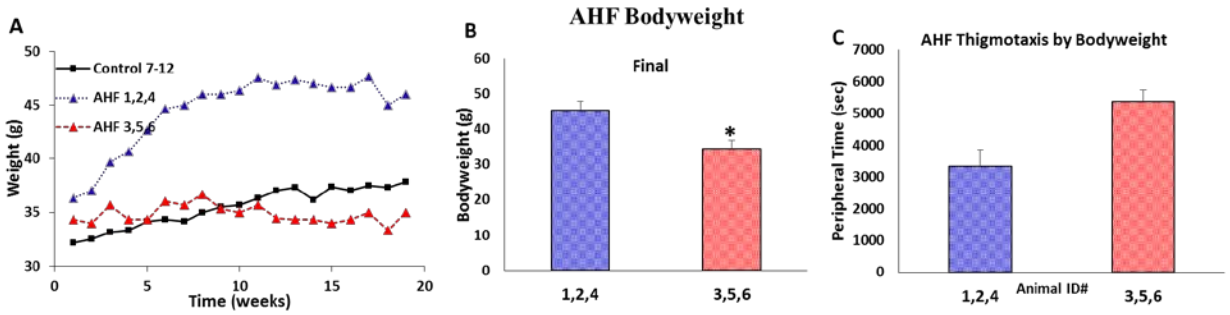

Figure 18. **Low bodyweight AHF mice display increased thigmotaxis**. Mice with lower bodyweight explored the open field periphery more than did the average to overweight bodyweight mice. This is an indication of increased anxiety in the low bodyweight mice.

3.8 Grooming events decrease in AHF mice treated with HC067047.

AHF mice in both vehicle and drug groups show a decrease in grooming events between the 38°C and the 44°C modified hotplate **(see Figure 19A)**. Control mice show little change in the number of grooming events between the 38°C and the 44°C modified hotplate. AHF mice show increases in freeze and rear events between the 38°C and the 44°C modified hotplate, while control mice increase in freeze events (**Figures 19B and 19C)**. These changes are not significant by t-test.

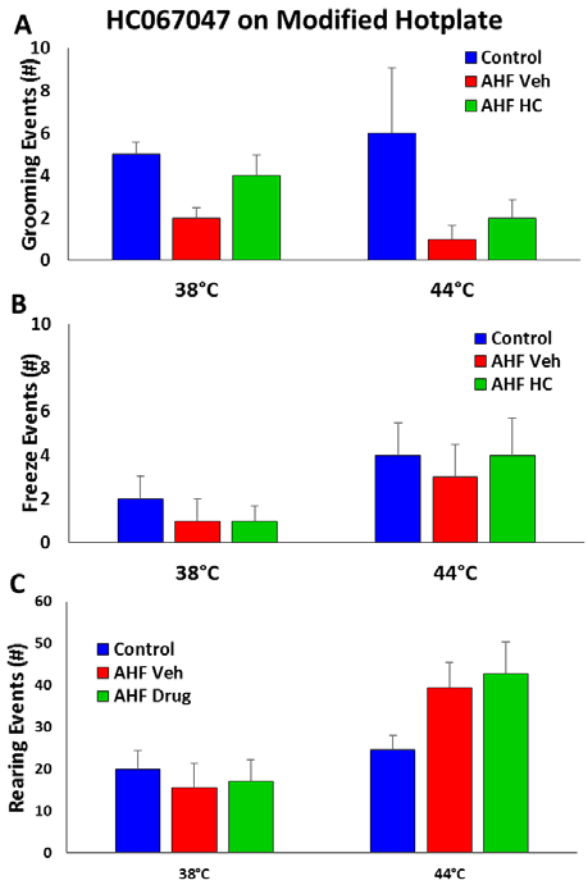

Figure 19. HC067047 affects anxiety- and fear-like behavior on the 44°C hotplate. (**A)** Grooming events decrease in AHF treated with HC 067047 on the 44°C modified hotplate versus the 38°C while grooming in control mice shows little change. (**B & C)** Both freezing and rearing events increase for AHF mice, while control mice show a definite increase in freeze events.

4. DISCUSSION

Significant differences between the AHF and control mice show that non-reflexive testing of AHF diet induced CP mice is valid. Non-reflexive testing allows for a more holistic view of the mouse study treatment groups, highlighting anxiety-like and fear-like behavior present in the animal. Anxiety interacts with pain report in the human clinical patient, therefore, it is absolutely necessary to include anxiety testing in a mouse model of human disease. A review of certain tests such as the plus maze and place preference tests in the battery results show that AHF mice show overall more anxiety-like behaviors, while displaying increased locomotion, both of which integrate with the increased exploration/escape responses seen in AHF mice. The AHF mice also show increased fear-like responses on the modified 44°C hotplate through increased Freeze events. In the open field AHF mice showed similar mobility

and decreased escape behaviors relative to controls. Testing with place preference, plus maze, open field, and the modified hotplate were done to better understand anxiety in CP.

4.1 Place Preference 44°C/22°C Escape Task – Transitions

Increased cumulative transitions on the 44°C/22°C Escape test by AHF mice indicate stress induced analgesia (SIA), likely due to greater starting hyperalgesia in the AHF group relative to controls. Escape testing forces mice to choose between an unpleasant and anxiety inducing brightly lit chamber, or a darkened 44°C hotplate chamber where the (heat induced) danger and attendant fear are clear and present (Rhudy and Meagher, 2000). Control mice choose to remain in the brightly lit chamber and experience anxiety-like behavior instead of the fear-like behavior (of heat damage) generated by the 44°C hotplate (Campos et al., 2013). AHF mice show signs of stress induced analgesia (SIA) which increases their latency to move out of the darkened chamber (off the 44°C plate).

The increase in transitions, suggestive of SIA, is striking as AHF mice transition a total of 33 times more than controls in the final week, and spend more time in the 44°C dark chamber than do control mice. Increased transitions and SIA in the AHF CP model beginning in week eight suggests that the model progresses from normogesic and non-stressed to hyperalgesic and stressed, perhaps as human CP patients do. This suggests that the model is translatable to human CP patients as these patients experience anxiety (Madan et al., 2013), fear (Fitzsimmons et al., 2005), and psychological components of the disease brought on by stress.

4.2 Place Preference 44°C/22°C Escape Task – Latency to transition

AHF mice display reluctance to transition between light and dark chambers in the seventh week likely due to increasing visceral hyperalgesia as CP begins. This behavior denotes an increase in anxiety-like behavior as the mice avoid entering the lit chamber, even though it

exposes them to algesic 44°C heat stimuli in the dark chamber. One interpretation of this behavior is that from an evolutionary standpoint, once a mouse is injured (visceral hyperalgesia begins) it is less able to escape from predators. Staying in the dark, even in the presence of algesic stimuli, would promote survival in an ill mouse. There is also the distinct possibility that SIA allowed the AHF mice to remain in the dark with the noxious heat longer before they had to remove themselves from the 44°C heat stimuli and expose themselves to light and the evolutionary anxiety posed by the danger of being seen by a predator. In support of this argument, significant SIA manifests as increased transitions occur in week 8, which correlates with increased von Frey mechanical hypersensitivity data from week 7 (see Chapter 2).

4.3 Light/Dark Place Preference Test - Transitions

AHF mice transition significantly more in the light/dark place preference test in week nine, indicating an increase in exploratory behavior due to the anxiolytic effects of stress induced analgesia. Increased stress on the mouse has been shown to trigger SIA, a form of endogenous analgesia, which can become conditioned to the test environment. The light/dark place preference test is typically considered an unpunished test (moving from lit chamber to darkened chamber includes no penalty). However, there is the possibility of contextual fear-like behavior induction here as the light/dark test apparatus used was also used for 44°C/22°C Escape testing, which does punish the mice when they attempt to seek the darkness as the darkened chamber floor is regulated to 44°C. Note that while both control and AHF animals were likely to experience stress upon placement in the test apparatus, the AHF mice entered testing with increased stress due to visceral hyperalgesia. AHF mouse visceral hyperalgesia stress is additive to that produced by testing.

4.4 Smooth or Rough Mechanical Surface - Grooming

The transition from smooth to rough surface affects anxiety-like behavior (grooming) and fear-like behavior (freezing) differently in Loperamide treated control and AHF CP mice, due to AHF mice hyperalgesia. Note that saline treated control mice, which represent the average untreated mouse, show a significant decrease in grooming (anxiety-like behavior) from the smooth to the rough surface. Both saline and Loperamide treated groups of AHF CP mice meanwhile show no significant difference on the smooth to rough surface, presumably since mice with visceral hyperalgesia have activated endogenous opioids. The endogenous opioids of the AHF CP mice would activate the same mu opioid receptors that Loperamide uses in an attempt to inhibit constant visceral pain. If the receptor is already occupied, then Loperamide would not further affect hyperalgesia. Also note that grooming latency increases significantly for both groups of control animals but not for AHF CP mice in the move from the smooth to the rough surface, indicating the anxiety-like behavior relieving action of Loperamide, but only in the control mice with intact, undamaged pancreas.

4.5 Smooth or Rough Mechanical Surface - Freezing

Freeze latency in Loperamide treated mice is significantly lower in AHF CP mice on the smooth surface. This latency decreases by about 16% (49 seconds) on the rough surface indicating that this continuous mechanical stimulation increases fear-like behaviors. Loperamide treated AHF mice on the rough plate also show a decrease in rearing (see Chapter 2) indicating decreased escape behaviors.

Table 7. AHF Mouse Fear-Like Behavior	Rough	Indication
Rearing (Events and Duration)	Decreased	Decreased escape behaviors
Freezing (Latency)	Decreased	Increased fear-like behaviors

Table 7 summarizes AHF mouse rearing and freezing data from **Figure 17**.

4.6 Plus Maze

In **Figure 15**, the increase in closed arm entries as well as open arm head entries of AHF CP mice relative to controls are indicative of increased exploration and anxiety-like behavior due to the effects of chronic visceral hyperalgesia. Plus maze pain model testing generally shows decreased closed arm entries, however, AHF mice show increased exploratory drive, possibly due to the effects of stress induced analgesia. SIA, just like in continued motion of injured human marathoners, causes the mice to continue to move quickly through the closed arms, even though an injured mouse would be expected to attempt to locate a dark place safe from predators. Once located the injured mouse would be expected to stay in this darkened area which is provided here by the closed arms, as the closed arms are darker than the center or open arms. The AHF mice are interested in exploring the open arms but are reluctant to do so due to their chronic hyperalgesia-induced anxiety-like behavior because they would be less able to quickly return to the safety of the dark arms.

4.7 Open Field Testing - Exploratory Behavior and Anxiety-like Behavior

As shown in **Figure 16**, AHF mice display decreased exploratory behavior on the open field test caused by increased anxiety-like behavior. Rearing (Crawley, 1985) and rearing duration are escape behavior indicators. Anxiety can cause decreased rearing (Neubert et al., 2007). Anxiety can also cause decreased rearing duration as anxiety-like behavior wins out over

escape behavior. A mouse, for instance, could be rearing when anxiety fueled innate predator avoidance behavior causes the mouse to drop out of the rear and into a more defensive position.

4.8 Open Field – Thigmotaxis

AHF mice in the low bodyweight group displayed increased anxiety-like behavior through thigmotaxis. Mice prefer dark, small, unexposed areas, likely due to evolutionary pressure leading to higher survival for mice that stayed hidden from the sight of predators (Campos et al., 2013). Anxious mice tend to spend more time exploring the periphery of the open field test (Simon et al., 1994). The low body weight AHF mice displayed greater stress than higher weight AHF mice, or control animals. Low bodyweight mice demonstrate thigmotaxis, the exploration of the periphery of the test chamber, due to increased stress. Less center time is an anxiety-like behavior.

4.9 Modified 44°C Hotplate with Loperamide – Freeze

As shown in **Figure 17**, freeze events are significantly increased in 1.2 mg/kg Loperamide treated AHF mice possibly due to contextual fear imbued in the hotplate setting. The hotplate setting can cause contextual fear-like behavior (Campos et al., 2013) and fear-like behavior is indicated when mice freeze. Conditioning of AHF mice by hotplate algesic stimuli causes fear-like behavior in AHF mice already stressed by visceral hyperalgesia. It is likely that "fear" of the hotplate algesic stimuli causes increased freezing in stressed AHF mice despite the analgesia typically provided by Loperamide.

4.10 Modified 44°C Hotplate with HC067047 - Grooming

AHF mice treated with HC067047, a TRPV4 antagonist, show decreased grooming events, indicating decreased anxiety-like behavior. Concurrently, both rearing (see Chapter 2) and freezing events increase suggesting that anxiety-like behavior is replaced with fear-like

behavior and exploration with intent to escape. The increase in rearing and freeze events may indicate that while HC067047 is an effective TRPV4 antagonist, it is less effective at inhibiting fear-related algesia behaviors experienced at 44°C (see Chapter 2). Except for increased freezing the control mice showed little to no shift in behavior suggesting that the HC067047 drug may have decreased algesic stimuli in the healthy controls. Control mouse increase in freezing could be attributed to contextual fear of the hotplate setting.

5. SUMMARY

Production of a dietary induced mouse model of AHF-induced CP without the use of harsh chemicals provides a more clinically translational model. A test battery including light/dark and 44°C/22°C place preference, plus maze, open field, and the modified hotplate can be used to better understand anxiety-like behaviors in a mouse model of chronic visceral pain. Testing in the CP model indicated that increased anxiety-like behavior develops after week 8, showing that non-reflexive testing is a valid indicator of chronic pain and anxiety. The AHF mice with CP display increased anxiety-like behaviors including increased exploration/escape responses and locomotion in the plus maze, and fear-like freezing behavior on the modified 44°C hotplate. In the open field test where mice explore spontaneously, AHF mice initially showed mobility and escape behaviors similar to controls, but continuation of the test promoted decreased escape behaviors. Thus, this study indicates AHF mice with CP have increased anxiety-like behavior, as do human patients who are diagnosed with CP pain, and thus provides a reasonable model to test pharmacological drugs to reduce anxiety accompanying chronic pain.

CHAPTER 4: Gerontological Considerations, Summary, and Conclusions

1 INTRODUCTION

The research presented in this dissertation, based on the production of this AHF mouse model of CP, show how long-term overconsumption of alcohol and a high fat diet can influence health span through decreased pancreatic health over the long term. A mouse model of CP induced only by consumption of a liquid diet with added alcohol and high in fat was the first task and composes Specific Aim One. Histopathology and hypersensitive behaviors indicated the production of the AHF CP mouse model, which will be presented first. Characterization of anxiety-like behavior in the AHF CP mouse model using both reflexive and non-reflexive testing methods was the second Specific Aim task. Anxiety-like and fear-like behavior were characterized in the model. Specific Aim 2 will be presented following Specific Aim 1.

This chapter provides a summary and conclusions of the research findings. One topic of the work is that consumption of the AHF diet in mice causes decreased pancreatic health manifesting as CP. In humans lifestyle changes can decrease the risk factors for damaging the pancreas through the AHF diet. Significantly decreasing or abstaining from alcohol, and eating a healthy diet reduce risk factors for CP development. Compression of morbidity is harder to obtain for a mouse or a human when consuming the AHF diet.

Physiologic reserve tends to decrease in normative aging. Chronic physical and mental pain decrease reserves further, which can lead to increased mortality at a younger age. Maintenance of health is diminished in populations such as alcoholic pancreatitis sufferers due to overconsumption of alcohol, and consumption of high fat foods. Alcohol and high fat foods can be comforting and assist in an individual's control of stress in their life (Gilman et al., 2008,

Tomiyama et al., 2011). Using alcohol and food to control mental anxiety would impact the physical body.

The specific aims will be discussed one at a time, followed by a summary of the results, and finally limitations for each chapter. Future research will then be discussed. The main findings of the work supporting the two experimental questions are summarized in Chapter 2 "A Mouse Model of CP Induced by an Alcohol and High Fat Diet", and Chapter 3 "Anxiety-like and Fear-like Behaviors in a Mouse Chronic Pancreatitis Model". See below for a synthesis of the main findings of the research that answer the two research questions of the study.

2 SPECIFIC AIM 1: PRODUCE THE ALCOHOL AND HIGH FAT CHRONIC PANCREATITIS MOUSE MODEL

The first aim of the research was to produce a CP mouse model induced only by an alcohol and high fat diet. This mouse model was expected to show both histopathology and pain-like behaviors indicative of CP. The AHF diet has been shown to reliably induce CP and pain-like behavior in rats (McIlwrath and Westlund, 2015, Zhang et al., 2015). This particular CP model has not been produced in mice but would allow for work in transgenic mice. Mouse genetic modification techniques are much more reliable than those for rats.

The modified Lieber-DeCarli diet was fed over a period of months during which AHF mice showed indications of increased hypersensitivity to algesic stimuli. AHF mouse pancreatic histopathology was also indicative of CP with increased fibrosis, atrophy, and diffuse nuclear material apparent. These histopathological indicators as well as hypersensitivity in the AHF mice indicate the presence of CP. Drugs were also tested to determine their effectiveness for relief of hypersensitivity in the AHF CP mouse model.

The study of CP in a non-chemical-induced mouse model is essential to understand the disease. The AHF diet induces the morbidity of CP in mice in a manner similar to the induction

of the morbidity of CP “process” in humans. The research data presented here, as well as the literature (Marmot, 2015), highlight the need for humans to make healthy lifestyle choices over time to maintain physiological reserve and health.

2.1 Chapter 2 Results

The AHF only induction of CP in a mouse model is a reality and exhibits face validity as the model displaying both histopathology (physiological) and hyperalgesia (behavior) common to CP.

Histopathology

a. Histopathology conducted on the pancreas of AHF pancreatitis mice showed morphology common to CP: fibrosis, diffuse nuclear material, and poor cellular geometry.

b. Percent fibrosis of pancreas in the AHF pancreatitis mice was significantly greater when compared to normal chow controls.

Hyperalgesia

c. AHF mice displayed mechanical hypersensitivity with a lower paw withdrawal threshold than controls on the von Frey test.

d. AHF pancreatitis mice showed significantly increased numbers of rearing events on the rough surface and the 44°C modified hotplate.

Drugs

e. Loperamide increased PWT in AHF pancreatitis mice significantly nearer to control values and produced a dose-dependent decrease in rearing events of the AHF pancreatitis mouse on the modified 44°C hotplate.

f. Treatment of AHF mice with TRPV4 antagonist HC067047 significantly prolonged the first jump latency, and the number of jump events decreased, on the 44°C hotplate.

2.2 Limitations of the Research

Access to funding, and specialized resources, as well as a limited time-frame influenced the research. There was a limited time frame to complete production and characterization of the AHF CP model, consequently certain questions had to be shelved for future research. Patients with CP tend to be smokers, who over-consume alcohol, and may eat high calorie foods (Yadav and Lowenfels, 2013). Enhancing the AHF diet with smoking could create a mouse model that more fully recapitulates the clinical etiology of human CP. Smoking may drive increased progression of disease in the mouse model. This AHF diet with smoking is not at all common in the literature, though it appears to be the most clinically relevant model. Note that Kentucky has one of the highest smoking rates in the country at up to 27% (CDC, 2015a). Smoking resources were enquired about but would have had time requirements beyond those available. Funding for this research was not available.

2.3 Future Research

2.3.1 Blood Alcohol Concentration

Blood alcohol concentration (BAC) is a measure of alcohol concentration in the blood. The BAC changes depending on how much and what type of alcohol has been consumed over a period of time (Cox et al., 1985, Fisher et al., 1987). Though blood samples were taken from the mice and processed into serum, a BAC alcohol analyzer was not available for testing samples. Future research should include BAC testing.

Blood alcohol concentration was estimated using the BAC formula in Winek and Murphy (1984). See Appendix A for calculations. The estimated blood alcohol level of the AHF mice is

between 0.021 and 0.086 percent at any given time. The AHF mice consumed different amounts of alcohol-enriched liquid food each day over the course of the study. At the low end of the range (BAC=0.021) humans may exhibit slight impairment (NHTSA, 2005). A BAC of 0.086 would result in a loss of balance and reaction time in humans (NHTSA, 2005). A BAC of 0.08 in a human is considered legally intoxicated (NHTSA, 2005). The AHF mice performed comparably to the control mice on the Open Field test, indicating no loss of locomotion (see chapter 3). AHF mice did not exhibit ataxia or other notable features on alcohol impairment possibly due to alcohol tolerance.

2.3.2 The Alcohol and High Fat Chronic Pancreatitis Model with Smoking

Patients with CP tend to be smokers, who over-consume alcohol, and may eat fatty foods (Yadav and Lowenfels, 2013). Enhancing the AHF diet with smoking could create a mouse model that mimics the etiology of human CP. An experimental timeline for such a study is proposed in **Figure 20**. Smoking may drive the faster progression of the AHF CP mouse model. This AHF diet with smoking is not at all common in the literature, though it appears to be the most clinically relevant model. Kentucky has one of the highest smoking rates in the country at up to 27% (CDC, 2015a). Resources to expose the AHF diet mice to smoking-like conditions were not immediately available. However, the experimental timeline is proposed.

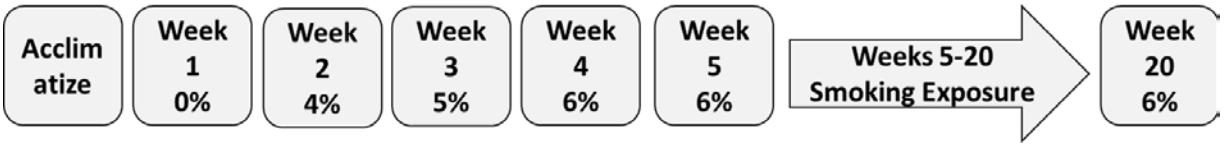

Figure 20. Alcohol and High Fat Chronic Pancreatitis Mouse Model with Smoking. Mouse cohorts acclimatize for one week then begin the Lieber-DeCarli liquid diet with added alcohol. Mice begin the diet at 0% alcohol and are stepped up gradually to the maintenance dose of 6%. Mice are exposed to “smoking” for fifteen weeks.

2.3.3 Rota-rod Physiological Reserve Testing

While physiological reserve is a good concept for mechanistic explanation of the work, specific testing of physiological reserve would improve the research. Future research will include testing of a measure of physiological reserve at baseline and throughout the duration of the research.

Rota-rod testing is one option for testing physiological reserve change progression across the course of the research. In accordance with PR, AHF mice would be expected to lose function on the rota-rod at a higher rate than control mice. A decrease in PR brought on by harmful drinking and high fat consumption allows hazardous risk factor inflicted damage mechanisms to take root and damage the pancreas. The decrease in PR in the case of the AHF CP mice allows the progression of CP.

3 SPECIFIC AIM 2: CHARACTERIZE ANXIETY-LIKE BEHAVIORS IN THE AHF CP MOUSE MODEL

The second aim of the research was to characterize anxiety-like behaviors in the AHF CP mouse model using non-reflexive testing methods. Anxiety behavior characterization in mouse models of CP is sparse. Anxiety has been shown to be active as part of a disease intensifying stress-depression-anxiety axis (Takano et al., 1992, Krantz and McCeney, 2002, Leserman et al., 2002, Roy-Byrne et al., 2008, Steimer, 2011). In humans, anxiety is often comorbid with pain and depression (Kroenke et al., 2013). The AHF CP mouse model was expected to show increased anxiety-like behaviors in non-reflexive testing relative to the controls. Increased anxiety-like and fear-like behaviors were detected in characterization of the AHF CP mouse model. Future work should include characterization of depression in the AHF CP mouse model because depression, anxiety, and chronic pain are interrelated in humans (Kroenke et al., 2013).

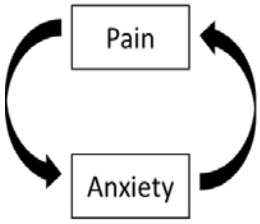

Figure 21. Pain and anxiety are interrelated. In humans, anxiety increases perception of pain intensity. Chronic pain, anxiety, and depression can be comorbid.

The modified Lieber-DeCarli diet was fed over a period of months during which AHF CP mice showed indications of increased anxiety-like behavior. AHF CP mice also showed fear-like behavior. HC067047 and Loperamide HCl drugs were tested to determine their effectiveness, or provide a positive control, for relief of hypersensitivity induced anxiety-like and fear-like behaviors in the AHF CP mouse model.

AHF CP mouse model testing included both reflexive and non-reflexive testing. Reflexive testing does not require input from the brain. Mouse reflex action is spinal cord mediated. Laboratory animal drug testing has traditionally been concerned with relieving acute pain stimuli such as that seen on the hotplate test. Acute testing tends to focus on pain carried by the Aδ afferents, also known as "first pain". First pain is immediate upon exposure to a painful stimulus. Descriptors of this pain in humans include "sharp" or "stabbing". Aδ fiber myelination allows quick movement of the afferent pain signal to the brain. Aδ fibers conduct signals at a speed of (5-30 m/s) to the brain.

Second pain is delayed pain felt after first pain subsides. Descriptors of this pain in humans include "dull" or "throbbing". Second pain is carried by small unmyelinated C-fibers. The lack of myelination means slow movement of the pain signal to the brain. The C-fiber carries signals at a rate of (0.5-2 m/s) to the brain.

Chronic pain is a significant problem for patients in the clinic. Chronic pain in the clinic can be associated with prolonged activation of the C-nociceptors (Yeomans et al., 1996). Slow increases of nociceptive stimuli such as heat tend to replicate a type of pain seen in humans in

the clinic as it activates C-fiber nociceptors preferentially. Non-reflexive test measures require the ascending nociceptive stimulus to travel through the spinal cord, to the brainstem, and the cerebral cortex before motor action is taken. Significant behavioral differences between the AHF CP and control mice show that non-reflexive testing of AHF diet induced CP mice is valid. Non-reflexive testing allows for a more holistic view of the mouse study treatment groups, highlighting anxiety-like and fear-like behaviors present in the animal.

3.1 Chapter 3 Results

Mice with AHF diet-induced CP show increased anxiety-like and fear-like behaviors.

Anxiety-like Behavior

a. A review of the plus maze and place preference tests show that AHF mice display overall more anxiety-like behavior, while displaying increased locomotion, both of which integrate with the increased exploration/escape responses seen in AHF mice.

b. AHF mice display decreased escape behavior compared to control mice on the open field test while AHF mice in the low bodyweight group displayed increased anxiety-like behavior through thigmotaxis.

Stress-induced analgesia (SIA)

c. SIA allowed the AHF mice to remain in the 44°C dark longer before they had to remove themselves from the algesic stimuli.

d. AHF mice transition significantly more in the light/dark place preference.

Fear-like Behavior

e. The AHF mice show increased fear-like responses on the modified 44°C hotplate through increased freeze events.

f. Freeze latency in Loperamide treated mice is significantly higher in AHF CP mice on the smooth surface. This latency decreases by 16% (49 seconds) on the rough surface indicating that this noxious mechanical stimulation increases fear-like behaviors.

g. The increase in rearing and freeze events may indicate that while HC067047 is an effective TRPV4 antagonist, it is less effective at inhibiting fear-inducing algesia behaviors experienced at 44°C.

3.2 Limitations of the Research

Gerontological limitations center on the physiological, mental, and social complexity inherent in people in comparison to mice. Whereas people are physically and genetically heterogeneous, especially as they age, inbred laboratory mice are bred to be homogenous. People have much more complex lives and brains than laboratory mice. We have and can describe emotions, we can report pain, especially where the pain is, how intense it is, and the duration of pain. Humans also have cognitive complexity, a rich variety of social opportunities, and personalities that can affect health. People possess preconceived notions about aging and pain, and have the ability to make choices based on their perception of the situation. People can choose to go to a bar or eat high fat foods. Mice do not have free will under experimental conditions. They cannot choose their actions beyond a certain point. Human brains are more developed than mouse brains, especially in the areas of speech and thought. Mice obviously cannot talk like humans can. This makes studying certain factors like suicidal tendencies in mice impossible. There are many interactions here that cannot be easily studied in mice, if at all. Perception, for example, cannot be studied on a mouse model. Perception, however, plays a part in human pain. Animals can be influenced by a context such as familiar patterns in a chamber of a test apparatus but perception can’t be studied in a mouse. Pain is composed of at least two

parts, the physical and the psychological. So pain itself cannot actually be studied in an animal model, only pain-like behavior.

An important limitation in scientific research involving animals is the presence of a control group of animals for comparison to the experimental group. This control group, unlike the experimental group, does not experience the introduction of an experimental variable. In the research conducted in this dissertation there was a treatment group as well as a control group. The control group in an animal experiment helps the scientist determine if changes seen in the treatment group are due to treatment (the AHF diet here) or to normal changes taking place. Control mice in this research showed significantly less pancreatic tissue fibrosis than did AHF CP mice. In addition, AHF CP mice showed atrophy and irregular cellular architecture (Zhang et al., 2014), while control mice did not. The group receiving treatment showed marked pancreatic difference from the control group. This research data suggests that the AHF CP mouse pancreatic changes were due to the AHF diet.

3.3 Future Research

3.3.1 Cognitive Impairment

AHF mice also did not appear cognitively impaired. It is of note however that mouse models of mild cognitive impairment (MCI) may not show locomotion changes (Pepeu, 2004). Cognitive impairment can manifest in a loss of weight in humans (Cronk et al., 2010). The AHF mouse group split into high and low bodyweight groups across the course of the research. Cognitive differences might have been apparent, if tested, especially in the low bodyweight AHF mice. Depression can be comorbid with cognitive impairment in humans (Bhalla et al., 2009).

Future studies should include testing for cognitive impairment as well as comorbid depression. Cognitive impairment testing would include the Morris water maze (MWM). The

MWM detects cognitive impairment in mice tasked to locate a platform located within a pool of water. Mice that take longer to locate the platform, or do not stay in the quadrant the platform has always been in when the platform is removed on the last day, are likely cognitively impaired.

3.3.2 Depression

As anxiety, pain, and depression are interrelated, testing for depression-like behavior would have added another dimension to the research. The forced swim test, splash test, and tail suspension test can reveal the presence of depression in mice (Santarelli et al., 2003, Cryan and Holmes, 2005). The forced swim test involves allowing a mouse to swim in a cylinder full of water until it stops swimming. The presence of depression can be determined if mice fail to groom after sucrose solution is sprayed on their tail base. In the tail suspension test, mice are suspended from their tail and observed.

4 THE HEALTH SPAN, HOMEODYNAMIC SPACE, & AHF CHRONIC PANCREATITIS MICE

Health span, the part of one's life free from certain morbidities and disability, is decreased by factors such as overconsumption of alcohol and high fat foods. These risk factors are seen in the literature for humans who develop CP (Yadav and Lowenfels, 2013). The health span view is well suited to research concerning the production and characterization of an AHF CP mouse model. This research employed a health span view as AHF CP mice were compared to control mice. AHF CP mice have, through application of the 6% alcohol Lieber-DeCarli liquid diet and high fat lard supplementation seen a decrease in health in relation to control animals.

The term homeostasis describes an attempt by the body to continually return to one set point, that remains the same, such as temperature, while homeodynamics covers the changes in this set-point as the body attempts to adjust to changes affecting its ability to adapt, its

homeodynamic space (Rattan, 2006). Chronic illness is indicative of a shrinkage and loss of homeodynamic space (Rattan, 2013, 2015). Homeodynamic space loss has been mitigated to some extent by the medical establishment. Medical treatments are available for those suffering from a variety of illnesses, and depending on the receptiveness and strength of the individual, can be quite effective against certain diseases. Many morbidities can be attenuated by exercise, good nutrition, stopping smoking, and keeping an eye on biomarkers of health such as blood pressure (Rowe and Kahn, 1998) p 17,41,78).

5 JUSTIFICATION FOR THE RESEARCH

The National Commission on Digestive Diseases (NCDD) under the umbrella of the National Institute of Diabetes and Digestion and Kidney Diseases (NIDDK), value the production of better animal models to further the study of alcoholic pancreatitis (Witt et al., 2007). Pain-like behaviors in CP animal models are a crucial area of much needed research and have also been called for (Reed, 2014). The present study contributes to the study of CP in mouse models by providing a nontransgenic mouse model of alcoholic CP induced by diet alone, without the use of chemicals such as cerulein or dibutyltin (DBTC) commonly used to induce rodent models of pancreatitis. The association of pancreatitis pathology with anxiety has been understudied. Anxiety, like pain, is useful as a transient state but when anxiety is prolonged it is termed a disorder. Anxiety is often comorbid with pain and depression (Kroenke et al., 2013). Therefore, it is important to determine anxiety in mice with CP histology.

6 FACE VALIDITY OF THE MODEL

It is necessary to produce and use models that mirror etiologically determined risk factors as these are the most likely to properly predict the human patient’s response to treatments. Nontransgenic mouse models where pancreatitis is induced only by a combination of ad libitum

liquid food with added alcohol and lard supplementation are rare and study of anxiety-like behaviors has not been conducted in this mouse model. It is critical to produce and use models with construct validity in order to increase the predictive validity of drugs studied to treat CP. More than 50% of pharmacological treatments do not pass phases II and III testing due to lack of efficacy (Arrowsmith, 2011a, b). While a useful method for some studies, chemical injection to create disease may not capture with accuracy all that occurs in the body over time with natural onset chronic pancreatitis. The AHF CP nontransgenic mouse model mimics disease induced by the risk factors of alcohol and high fat diet consumption found in humans with CP and provides construct and face validity, two factors important in predictive validity.

7 PROS AND CONS OF CERULEIN OR DBTC PANCREATITIS INDUCTION

Current rodent models of pancreatitis induced by chemical irritants such as cerulein or dibutyltin dichloride (DBTC) have proven fruitful for studies of acute pancreatitis. Cerulein injections expose the pancreas to supraphysiologic cholecystokinin (CCK) levels, which are not associated with the development of CP in humans, but do produce AP symptoms in mice (Reed, 2014). Cerulein treatment, a common and reproducible method for induction of AP in mice, causes repeated damage to the pancreas as repeated injections are given, creating the cycle of damage to the pancreas like repeated human heavy alcohol use (Reed, 2014). DBTC treatment is a less used method as it is highly toxic, difficult to use in inexperienced hands, and like acute pancreatitis in patients it causes mortality in the process of disease development. Disease development occurs at a rate of about 33% in treated animals (Reed, 2014). In addition, DBTC does not cause CP in humans (Reed, 2014). The alcohol and high fat chronic pancreatitis mouse model more naturally recapitulates the etiology of alcoholic CP seen in humans.

8 SIMILARITY OF MOUSE RESEARCH TO ETIOLOGICAL RISK FACTORS OF ALCOHOLIC CHRONIC PANCREATITIS

The AHF diet is similar to the overconsumption of alcohol (Irving et al., 2009, Yadav and Lowenfels, 2013) and high fat diet seen in humans that develop alcoholic chronic pancreatitis. Production of a mouse model of AHF-induced CP without surgery or injected chemicals provides a more etiology-based model. The AHF CP model exhibits construct validity in that CP is induced by a diet including high fat and alcohol. Human etiological risk factors for CP include alcohol abuse as heavy drinkers have 4X the risk of developing CP (Yadav et al., 2007a), while obesity, indicative of a high fat diet, increases the risk of developing CP by 2X (Sadr-Azodi et al., 2013). The AHF CP mouse regularly consumes alcohol (6%) and a high fat diet (57%).

9 MODEL ENABLES FURTHER RESEARCH

The induction of CP with the AHF diet follows the natural known etiology of induction in humans diagnosed with CP. In addition, the production of this model in mice allows for the future use of transgenic mice and drugs to further the study of alcoholic pancreatitis and associated conditions. This model also will allow for study of the effects of anxiety on pancreatitis histology.

10 CONCLUSIONS

10.1 Of Mice and Men

The AHF diet model in mice is a useful model of human CP with many advantages for experimental studies. The AHF diet induces the morbidity of CP in mice likely in a similar manner as for the induction of the morbidity of CP “process” in humans. Like selected humans, older male mice that consume an AHF diet develop CP.

10.2 Healthy Dietary Choices in Two Populations

Older male mice that do not consume an AHF diet appear healthy, with bright eyes and sleek fur. Control mice were in better condition than AHF mice like people who make lower fat food and moderate to no alcohol part of their lifestyle. Control mice, like a healthy population of people, may have lived natural lives to older old age in relatively good health while AHF mice, like an unhealthy population die young.

10.3 Pain in Two Populations

AHF mice displayed hypersensitivity not seen in control mice. Pain in humans significantly decreases quality of life (Fitzsimmons et al., 2005, Torrance et al., 2010). Control mice may have seen homeodynamic space shrinkage but AHF mice were in poor condition for a long time and appeared to have homeodynamic space shrinkage.

10.4 Health in Two Populations

Dietary choices that in humans can lead to the morbidity of CP such as overconsumption of alcohol & a high fat diet (Kushner et al., 2005, Irving et al., 2009, Sadr-Azodi et al., 2013, Yadav and Lowenfels, 2013), parallel those seen in alcohol and high fat mice that lead to induction of CP. Healthy dietary and lifestyle habits can decrease human morbidity (Maxmen, 2012), and increase human population maintenance of homeodynamic space. Personal choices by humans about diet and alcohol use can affect individual and population health. Negative dietary choices can lead to loss of health in the population making these choices. Positive dietary choices can lead to maintenance of homeodynamic space.

APPENDIX A

8 grams Liquid Diet with 6% EtOH

BAC = (150/bodyweight)(% EtOH/50)(ounces consumed)(0.025)

BAC= (150/0.0882 lbs)(6% EtOH/50)(0.01693 oz EtOH)(0.025)

BAC= (1700)(0.12)(0.01693)(0.025)

BAC= 0.086

2 grams Liquid Diet with 6% EtOH

BAC = (150/bodyweight)(% EtOH/50)(ounces consumed)(0.025)

BAC= (150/0.0882 lbs)(6% EtOH/50)(0.0042 oz EtOH)(0.025)

BAC= (1700)(0.12)(0.0042)(0.025)

BAC = 0.021

Figure 22. Calculating estimated blood alcohol concentration (BAC). Average weight of mice at study termination was 40 g which was converted here to pounds. The ethanol concentration was 6% in the prepared liquid diet. The average grams of alcohol consumed was converted to ounces. The average liquid food consumption was 8 grams/day, however, mice consumed as little as 2 grams/day. BAC range is calculated between 0.021 and 0.086 (Winek and Murphy, 1984).

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Zhang L, Zhang X, Westlund KN (2004) Restoration of spontaneous exploratory behaviors with an intrathecal NMDA receptor antagonist or a PKC inhibitor in rats with acute pancreatitis. Pharmacology, biochemistry, and behavior 77:145-153.

Zhang LP, Kline RHt, Deevska G, Ma F, Nikolova-Karakashian M, Westlund KN (2015) Alcohol and high fat induced chronic pancreatitis: TRPV4 antagonist reduces hypersensitivity. Neuroscience 311:166-179.

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Zhang X, Cui Y, Fang L, Li F (2008) Chronic high-fat diets induce oxide injuries and fibrogenesis of pancreatic cells in rats. Pancreas 37:e31-38.

Curriculum Vitae

Tiffanie Clinkinbeard

Education

1999-2002 BS Biology, Chemistry Minor, Christopher Newport University

Research Interests

I am interested in the differential effect of genetics and stress on lifespan, particularly as they pertain to maintaining good health throughout life. Research areas of interest include neurodegeneration, pain, anxiety and fear, insulin sensitivity, endoplasmic reticulum stress activation of the unfolded protein response (UPR), the mammalian target of rapamycin (mTOR) pathway, cellular adaptation to stress and effects on health, transcription and translation control in various stress conditions, genetic variation such as single nucleotide polymorphisms (SNP)'s and their effects on disease, prion-initiated diseases and other protein dysregulation diseases such as Alzheimer's Disease, micro RNA in disease phenotype and cellular stress response, induction of apoptosis in chronically stressed cells, sleep dysregulation as a cellular stressor, diet in cell signaling responses and longevity, pancreatitis, stroke, changes in the aforementioned areas in aged versus younger animals, as well as in different strains of the research model organism and the effects on disease presentation.

Work Experience:

1996-1999 Heavy-Wheeled Vehicle Mechanic, US Army (Honorable Discharge)
2000-2004 Battalion Supply Specialist, US Army Reserves
2003-2004 Animal Care Technician, Lexington VA Hospital, KY
2004-2008 Research Assistant, Valvoline New Product Development Laboratory, Lexington, KY
2008-2012 Research Assistantship, Graduate Center for Gerontology

Membership:

2002 Alpha Chi Honor Society
2008-2013 Sigma Phi Omega, Gamma Mu, University of Kentucky
2009-2014 Bluegrass Society for Neuroscience
2010 Gerontological Society of America
2011-Present Society for Neuroscience

Awards:

Military Army Service Ribbon
Military Army Achievement Medal
Military Good Conduct Medal

Service:

2011-2012 Graduate School Student Advisory Diversity Committee
2012-2013 Vision Committee, Graduate Center for Gerontology
2012-2013 Treasurer, Sigma Phi Omega, Gamma Mu, University of Kentucky

Publications:
Bardach S, Gayer C, **Clinkinbeard T**, Watkins J, Zanjani F. (2010) "Using a positive aging intervention to explore the malleability of possible selves and expectations regarding aging". Educational Gerontology, Vol. 36 Issue 5, p407-424

Clinkinbeard TC, Sarbani G, Craddock S, Pettigrew LC, Guttmann RP. (2013) "Calpain cleaves methionine aminopeptidase-2 in a rat model of ischemia". Brain Research, 1499, p129-135

Invited Presentations:

Clinkinbeard T. (Jan, 2013) "Live Long, Live Well" The Golden Girls, Lexington, KY

Presentations:

Clinkinbeard TC, Schoch K, Saatman K, Guttman RP. (April 11, 2011). "Calpain-mediated control of MetAP2 in Traumatic Brain Injury". Keeneland Conference for Public Health Systems and Services.

Clinkinbeard TC, Ghoshal S, Craddock S, Pettigrew LC, Guttmann RP. (April 17, 2012). "Calpain cleaves methionine aminopeptidase 2 in a rat model of ischemia." Keeneland Conference for Public Health Systems and Services.

Clinkinbeard TC, Ghoshal S, Craddock S, Pettigrew LC, Guttmann RP. (March 29, 2012). "Calpain cleaves methionine aminopeptidase 2 in a rat model of ischemia." Bluegrass Society for Neuroscience Spring Neuroscience Day Conference.

Clinkinbeard TC, Ghoshal S, Craddock S, Pettigrew LC, Guttmann RP. (October, 2012). "Calpain cleaves methionine aminopeptidase 2 in a rat model of ischemia." Society for Neuroscience Annual Conference.